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Characterisation of a post-entry restriction to HIV in human cells

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Submitted to the University of London for the degree of Doctor of Philosophy

November 2005

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Abstract

HIV-2 infected monocyte derived macrophages (MDM) at much lower efficiency compared to HIV-1 or to infection of PBMC. After a brief initial burst of replication, HIV-2 viral production was terminated. HIV-1 however rebounded cyclically in culture over a period of 20 days. Early entry events of HIV-2 in MDM were accommodated efficiently and replication could be restimulated with LPS indicating that HIV-2 enters a latent phase in MDM. The amino acid charge of the HIV-1 V3 loop is negatively correlated with macrophage tropism (Zhong et al., 1995), and I demonstrate that the HIV-2 V3 loop charge is also negatively correlated with MDM tropism, albeit weakly. This led me to investigate other determinants of HIV cellular tropism. An HIV-2 primary isolate that is unable to replicate in MDM was molecularly cloned (MCR) and the restriction (termed Lv2) mapped to virus *env* and *gag* (Schmitz et al., 2004). I show that a variety of primary HIV-1 and HIV-2 viruses are susceptible to Lv2 restriction.

Lv2 is a post-entry, post-reverse transcription restriction to HIV infection. To incorporate a role for Env, which acts at the cell surface, a model where the Env delivers a susceptible Gag into a restrictive cellular compartment was developed (Schmitz et al., 2004). The compartmentalisation model of Lv2 was tested using compounds that affect endocytic pathways (hypertonic sucrose) and lipid rafts (Methyl- β -cyclodextrin) in restrictive cells. With these methods I show that restricted virus can be rescued from Lv2 if a lipid raft-dependent endocytic pathway is inhibited. Fusion of restricted virus into HeLa/CD4 cells containing a tailless CD4 that located outside lipid rafts was fully permissive. The restrictive pathway was further defined using dominant negative mutants that specifically inhibit defined endocytic pathways; clathrin, caveolae and non-clathrin non-caveolae mediated. A role for an Arf6 non-clathrin non-caveolae mediated endocytic pathway in Lv2 restriction was demonstrated. Lastly, *env* swapped viruses demonstrate that delivery to the restriction pathway is Env dependent. In keeping with the Lv2 model, the unrestricted virus was unaffected by any of these treatments. Thus the route of entry, determined by the viral Env, can influence cellular tropism by avoiding intracellular blocks to infection.

The saturable nature of Lv2 restriction was investigated. HIV-2 Env pseudotypes of B- and N-tropic MLV pseudotypes saturated Lv2. This observation led me to investigate if human tripartite motif protein (TRIM) 5 α and members of the human TRIM family of proteins, shown to restrict MLV, mediated Lv2.

The TRIM family of proteins have been implicated in retroviral restrictions and even so far as to have broad anti-viral activity (Nisole et al., 2005). By choosing TRIM proteins with SPRY(B30.2) domains, and an RNA interference (RNAi) screening approach, TRIMs 1, 18 and 34 were identified as having Lv2 restriction activity. These proteins are expressed in restrictive HeLa/CD4 cells but not in permissive ones, thus acting as intracellular determinants of retroviral cell-tropism.

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Abbreviations

Ab	Antibody
Ad	Adenovirus
ADCC	Antibody dependent cell cytotoxicity
ADE	Antibody dependent enhancement
Ag	Antigen
AGM	African green monkey
AIDS	Acquired immune deficiency syndrome
AP	Alkaline phosphatase
APC	Antigen presenting cell
APOBEC	Apolipoprotein B mRNA-editing enzyme catalytic polypeptide
Arf-6	Adenosine ribosylation factor 6
ARM	Rev arginine-rich RNA binding motif
ARP	AIDS reagent program
AZT	3'-azido-3'-deoxythymidine
BAF	barrier to auto-integration factor
BrdU	Bromo-deoxy-Uridine
CA	Capsid
CAF	CD8 T cell antiviral factor
Cav-1	Caveolin-1
CCR	CC chemokine receptor
CD	Cluster of differentiation
CDK9	Cyclin dependent kinase 9
CMV	Cytomegalovirus
CNS	Central nervous system
Col	Colobus monkey
cPPT	Central polypurine tract
CPZ	Chimpanzee
CRF	Circulating recombinant forms
CTL	Cytotoxic T cell
CTS	Central termination sequence

CTxB	Cholera toxin binding subunit
CXCR	CXC chemokine receptor
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific ICAM-3 grabbing non-integrin
DMEM	Dulbeccos Modified Eagles medium
DMSO	Dimethyl sulphoxide
Dyn	Dynamin 2
EDTA	Ethylenediaminetetraacetic acid
EEA1	Early endosome antigen 1
EED	Embryonic ectoderm development protein
eGFP	Emerald green fluorescent protein
EIAV	Equine infectious anaemia virus
ELISA	Enzyme linked immunosorbent assay
ENV	Envelope
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complex required for transport
FCS	Fetal calf serum
FDC	Follicular dendritic cell
FFU	Focus forming units
FFU/ml	Focus forming units per ml
FITC	Fluoroscene isothiocyanate
Fv1	Friend virus restriction 1
Fv4	Friend virus restriction 4
GAG	Group specific antigen
GAP	GTPase activating proteins
GEF	Guanosine nucleotide exchange factor
HAART	Highly active anti-retroviral therapy
hAPOBEC	Human APOBEC
HFV	Human foamy viruses
HHV8	Human herpes virus 8
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen

HMGA1	High mobility group protein A1
hr	Hour
HS	Human serum
HSP 60	Heat shock protein 60
HSPG	Heparin sulphate proteoglycans
HTLV	Human T cell lymphotropic virus
huTRIM	Human tripartite motif
IDU	Injecting drug users
IFN	Interferon
IgG	Immunoglobulin G
IKB	Inhibitor of kappa B
IL	Interleukin
IN	Integrase
Ini 1	Integrase interactor protein 1
KS	Kaposi's sarcoma
LC	Langerhans cell
LEDGF	Lens epidermal growth factor
LTR	Long terminal repeat
Lv1	Lentivirus restriction 1
Lv2	Lentivirus restriction 2
MA	Matrix
mAb	Monoclonal antibody
Mac	Macaque
MCN	Molecular clone not restricted
MCR	Molecular clone restricted
MDM	Monocyte derived macrophages
MDTF	<i>Mus Dunnii</i> tail fibroblasts
MHC	Major histocompatibility complex
ml	Millilitre
MLV	Murine leukaemia virus
MLV-A	Amphotropic MLV (envelope/tropism)
MLV-B	B-tropic MLV

MLV-Mo	Moloney MLV
MLV-N	N-tropic MLV
MØ	Macrophage
MOI	Multiplicity of infection
MPV	Murine papillomavirus
MTOC	Microtubule organising centre
MVB	Multivesicular body
MβCD	Methyl-beta-cyclodextrin
Nab	Neutralising antibodies
NC	Nucleocapsid
Neo	Neomycin
NF-KB	Nuclear factor kappa B
NK	Natural killer cell
NLS	Nuclear localisation signal
NNRTI	Non-nucleotide reverse transcription inhibitor
NPC	Nuclear pore complex
NRTI	Nucleotide reverse transcription inhibitor
pAb	Polyclonal antibody
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHA	Phytohaemagglutinin
PIC	Preintegration complex
PM	Plasma membrane
PML	Promyelocytic leukaemia protein (TRIM 19)
PMPA	(R)-9-(2-phosphonylmethoxypropyl)
POL	Polymerase
PR	Protease
P-TEFb	Positive transcription elongation factor B
QPCR	Quantitative PCR
R5	CC chemokine receptor 5 using HIV
Rab	Rat brain

Rad 18	Radiation sensitivity protein 18
RANTES	Regulated on activation, normal T cell expressed and secreted
Ref1	Rebecca Fernley factor 1
REV	Regulator of expression of viral proteins
rhTRIM	Rhesus macaque tripartite motif
RNase H	Ribonuclease H
RPMI	Roswell park memorial institute
RRE	Rev response element
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
s	Seconds
SD	Splice donor
SDM	Standard deviation of the mean
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
siRNA	Short interfering ribonucleic acid
SIV	Simian immunodeficiency virus
SMM	Sooty mangabey
SP1	Spacer peptide 1
SP2	Spacer peptide 2
SSSV	Salmon swim bladder sarcoma virus
SU	Surface subunit of viral Env
SV 40	Simian virus 40
SYK	Sykes monkey
TAE	Tris-acetate EDTA
TAR	Transactivation response element
TAT	Transactivator of TAR
TBS	Tris buffered saline
TCID ₅₀	Tissue culture infectious dose 50
TCLA	T cell line adapted
TDF	Tenofovir disoproxil fumarate
T _h	T helper cell
TM	Transmembrane domain of viral Env

Trf	Transferrin
TRIM	Tripartite motif
TSG 101	Tumour suppressor gene 101
U	Units
V	Volts
V3	Variable loop 3
VIF	Viral infectivity factor
VPR	Viral protein R
VPU	Viral protein U
VPX	Viral protein X
VSV	Vesicular stomatitis virus
X4	CXC chemokine receptor 4 using HIV
Zeo	Zeomycin

Chapter 1

Introduction

1.1 Acquired Immune Deficiency Syndrome

In 1981, several patients were identified who had an insidious immune deficiency . The patients presented with opportunistic infections not seen before in the immune-competent, such as: oral Candidiasis, Kaposi Sarcoma (KS), Toxoplasmosis, *Pneumocystis carinii* pneumonia (PCP), and Cytomegalovirus (CMV) retinitis (Holland et al., 1982; Horowitz et al., 1983). After two years of infection, the case-fatality rate was approximately 90% and the cause of death in these immune compromised patients was often PCP, normally only seen in immunocompromised patients (Gottlieb et al., 1983; Groopman and Gottlieb, 1983; Murray et al., 1984). Immunologically, the majority of infected individuals appeared normal except for a reduction in their helper T cell subset. The percentages of B cells, circulating immunoglobulin levels, and natural killer (NK) and antibody-dependent cell-mediated cytotoxic T cell (ADCC) functions were normal, however there was a reduction in the patient CD4+/CD8+ T cell ratio. Therefore the cause of the immune deficiency appeared to be a loss of CD4+ T cells (Gottlieb et al., 1981; Schroff et al., 1983).

1.2 The discovery of the Human Immunodeficiency Viruses type 1 and type 2

In 1983 a T-Lymphotropic virus was isolated from a patient suffering from lymphadenopathy (Barre-Sinoussi et al., 1983). Before this discovery there were several hypotheses put forward for the cause of the growing number of lymphadenopathy cases, these included drug use, spermatozoa exposure, and even the use of amyl-nitrates, or 'poppers' that enhance sexual prowess (Gottlieb et al., 1981; Quagliarello, 1982). However, an overwhelming amount of evidence suggested an infectious aetiology, in particular, a blood transmitted infectious agent. It became apparent that not only homosexuals were susceptible to this new acquired immunodeficiency, but cases of infected intravenous drug users, haemophiliacs and

heterosexuals were becoming increasingly apparent (Evatt et al., 1984; Feorino et al., 1984; Griffin, 1983). Since a characteristic of the immune deficiency was the loss of the T-helper cell subset it was suggested that a T-Lymphotropic virus was responsible for the onset of AIDS, possibly even a member of the HTLV retrovirus family (Gallo et al., 1983). After sequencing and genotyping it was found that the AIDS virus resembled a member of the lentivirus family rather than the HTLV family of delta retroviruses and was termed the Human Immunodeficiency Virus type 1 (HIV-1) (Alizon and Montagnier, 1986; Coffin, 1986).

The Human Immunodeficiency Virus type 2 (HIV-2) was isolated in 1986 from two AIDS patients in West Africa. Then named Lymphadenopathy virus 2 (LAV-2), the envelope (Env) of HIV-2 reacted with serum from a macaque infected with STLV-IIImac (now known as SIV_{mac}) but not with serum from patients with HIV-1 (Barin et al., 1985; Chen et al., 1997; Clavel et al., 1986a; Clavel et al., 1986b; Clavel et al., 1987; Hahn et al., 2000). The sero cross reactivity of HIV-1 and HIV-2 was restricted to the core protein p24 (Barin et al., 1985; Clavel et al., 1986a; Clavel et al., 1987; Hahn et al., 2000). This suggested that the new virus, HIV-2, was more closely related to the simian virus than to HIV-1 [reviewed in (Hahn et al., 2000)]. The subsequent molecular cloning of HIV-2 suggested that it was distinct from both SIV and HIV-1 and more closely related to SIV (Clavel et al., 1986a).

1.3 Epidemiology of HIV-1 and HIV-2

By the end of 2004, approximately 58 million people had died of AIDS or were living with HIV infection (UNAIDS, 2004). The region with the greatest number of people infected with HIV is sub-Saharan Africa. The number of those currently infected in this region (December, 2004) stands at approximately 25 million, with Botswana (37.3% adult population as of end 2003), Lesotho (28.9% adult population as of end 2003), Swaziland (38.8% adult population as of end 2003), and South Africa (21.5% adult population as of end 2003) having the highest percentage of the population infected (UNAIDS, 2004).

1.4 Disease course and mechanism of T helper cell loss

1.4.1 Disease course

The first few weeks of HIV infection are typified by flu-like symptoms (“acute” in fig 1.1) that are coincident with a high plasma viral load. An adaptive immune response controls acute viraemia and restores CD4⁺ T cell levels, but does not eradicate the virus. High virus turnover depletes CD4⁺ T cell levels gradually over the course of a few years. When the CD4 count falls below 500 CD4⁺ T cells/ μ l opportunistic infections become more frequent, (mean of approximately 10 years) and an individual is considered to have AIDS when their CD4 count falls below 200 cells/ μ l (fig 1.1) (Castro et al., 1992; Janeway et al., 2001).

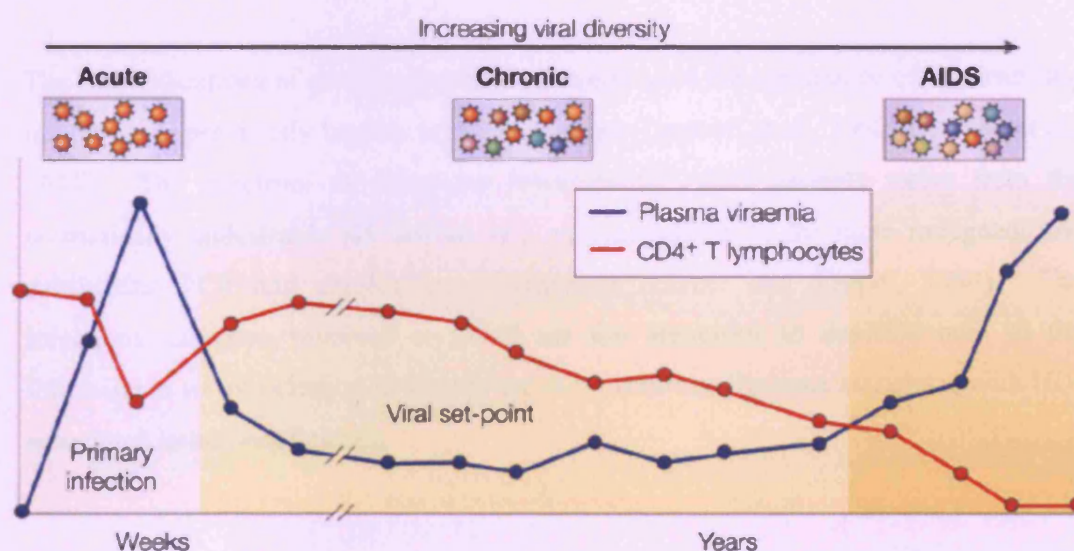


Figure 1.1. Disease course: CD4 count versus viraemia.

After the acute viraemia stage the CD4 count stabilises with the viral set point. The protracted chronic phase is clinically “silent” however there is very high virus turnover followed by depleted CD4 counts and a high level of viraemia with the onset of AIDS. From (Simon and Ho, 2003).

1.4.2 Mechanism of T cell loss

HIV preferentially infects HIV specific (Douek et al., 2002), and non-specific T_H cells (Badley et al., 2000). Autologous T cell death due to toxicity of direct infection is

thought to be just one of many mechanisms of T cell depletion [reviewed in (Simon and Ho, 2003)]. HIV induced enhancement of Fas ligand, TRAIL receptor, and even ligation of CD4 by gp120 have been shown to be a cause of bystander cell death [reviewed in (Badley et al., 2000; Simon and Ho, 2003)]. All of the above are thought to lead to CD4 counts less than 200 cells/ μ l that is the hallmark of AIDS (fig 1.1).

1.5 AIDS associated illnesses

T_H cell loss is associated with the onset of opportunistic infection. When circulating $CD4^+$ T cell levels fall to less than 500 cells/ μ l HIV infected individuals may start to present with opportunistic infections (Castro et al., 1992). The combination of declining $CD4^+$ T cells less than 200 cells/ μ l with or without presentation of opportunistic infections is a characteristic of AIDS (Castro et al., 1992).

The first indications of an emerging HIV epidemic were the appearance of opportunistic infections in previously healthy homosexual men (Gerstoft et al., 1982; Gottlieb et al., 1981). The spectrum of infections presented by AIDS patients varies from the cosmetically undesirable KS lesions and oral candidiasis to the more malignant and debilitating PCP and atypical mycobacteriosis (Lerner and Tapper, 1984). The infections/pathogens involved in AIDS are too numerous to describe here so the following is an introduction to four of the more common illnesses associated with HIV associated immunodeficiency.

1.5.1 Kaposi's sarcoma

KS lesions are often the first manifestation of AIDS and appear when the level of immunosuppression is still mild (Beral, 1991). Originally described by Moritz Kaposi in 1872 (Kaposi, 1872), KS is 20,000 times more common in those with AIDS than the general population (USA) and 300 times more common than in other immunosuppressed groups (Beral et al., 1990). There is also a correlation between KS and HIV infection in South Africa (Sitas et al., 1999). KS lesions are a tumour of vascular origin that tend to be brownish-purple and favour the extremities (Ruszczak et al., 1987; Samaniego et al., 1995), and are caused by human herpes virus 8 (Boshoff et

al., 1995; Chang et al., 1994; Dupin et al., 1999; Sirianni et al., 1997; Talbot and Crawford, 2004).

1.5.2 *Candida*

Opportunistic infection patterns vary from region to region, fungal infections were the most common opportunistic pathogen in those suffering from AIDS in the USA in 1981 (Gottlieb et al., 1983; Gottlieb et al., 1981). Primarily the *Candida* species, these infections can become disseminated and are becoming increasingly drug resistant (Coleman et al., 1998; Walsh and Groll, 1999). Oropharyngeal candidiasis caused by *Candida albicans* is an early indicator of AIDS that can become systemic if left untreated (McCullough et al., 1996). Indeed, the study of fungal infections of AIDS patients has led to the discovery of new pathogenic *Candida* species (Coleman et al., 1998; Sullivan et al., 1995).

1.5.3 *Pneumocystis carinii* pneumonia

Deaths of young homosexual men and injecting drug users (IDUs) in the early 1980s by PCP were some of the first indications that an epidemic of acquired immunodeficiency was emerging (Masur et al., 1981; Masur et al., 1982). *Pneumocystis carinii*, once thought to be a protozoan is now considered a fungus and causes acute “aggressive” pneumonia in immunodeficient humans, and *Pneumocystis* spp have been reported to cause disease in immunodeficient animals (Bartlett and Smith, 1991; Stringer, 1996). It is a major cause of “life-threatening” pneumonia and occurs in up to 80% of AIDS patients in the USA (Bartlett and Smith, 1991), making it the most common opportunistic infection in these patients [reviewed in (Sepkowitz, 2002)]. There is however a regional discrepancy; the incidence of PCP is only about 7% in African AIDS patients (Bartlett and Smith, 1991).

1.5.4 Typical and atypical Mycobacteriosis

A major cause of death in AIDS patients is the atypical Mycobacterial species. Disseminated *Mycobacterium avium* and CMV infections have been reported to be a leading cause of death in AIDS patients in the USA (Lerner and Tapper, 1984).

“Without HIV, the (*Mycobacterium*) tuberculosis epidemic would be in decline” (Nunn et al., 2005). Tuberculosis is the second leading cause of death world-wide and the leading cause of death of HIV patients in Africa (Frothingham et al., 2005; Nunn et al., 2005).

1.6 The immune response to HIV infection

1.6.1 Interferon response

A type 1 (cell mediated) immune response has been associated with suppression of viral load and long-term non-progression, with high IL-2 and IFN γ levels as strong correlates of maintenance of healthy CD4 cell counts (Clerici et al., 1996a; Salvaggio et al., 1996). Indeed, high IL-10 levels, indicating a switch to a predominantly humoral response (type 2), have been associated with a poor prognosis (Clerici et al., 1996b; Clerici et al., 1994).

1.6.2 $\gamma\delta$ T cells

The V γ V δ 2⁺ T cells recognise small organic phosphate antigens (Ag) and alkyl amines from a variety of bacterial and protozoal parasites without the need for MHC presentation or Ag processing (Janeway et al., 2001). There is no evidence that HIV encodes peptide Ags that are recognised by V γ V δ 2⁺ T cells (Chen and Letvin, 2003). However, the V γ V δ 2⁺ T cells can inhibit HIV replication *in vitro* because β -chemokines (MIP1- α , MIP1- β , and RANTES), shown to block entry of CCR5 (R5) using HIV viruses, are produced by phospho-Ag stimulated V γ V δ 2⁺ T cells (Poccia et al., 1999).

1.6.3 Natural Killer cells

Natural killer (NK) cells comprise about 15% of the peripheral blood lymphocytes, display a CD56⁺CD3⁻ phenotype and do not express specific Ag receptors like B and T cells. They are considered the “first line of defense”, secreting cytokines and chemokines upon activation, modulating other immune cells (Jacobs et al., 2005). Low

NK cell numbers in the blood of HIV-infected individuals are associated with a more rapid progression to disease that has been suggested to indicate the importance of NK cell functions in controlling HIV infection (Jacobs et al., 2005). HIV-1 accessory genes Vpu and Nef down-regulate MHC-I from the cell surface of HIV infected cells, hindering immune recognition and clearance by cytotoxic T-lymphocytes (CTL). However NK cells are able to recognise these MHC-corrupted cells and lyse them (Martin et al., 2002b).

1.6.4 CD8⁺ cytotoxic T cells

CTL (CD8⁺ V α V β T cells) detect virally infected cells by recognition of foreign peptides associated with MHC-I. Detection of foreign peptide leads to lysis and apoptosis of the infected cell by CTL secreted perforin and granzyme, respectively. Professional antigen presenting cells (APCs) are needed for optimal activation and expansion of CTL (Collins, 2004). The importance of CTL in controlling HIV viral load is underlined by the coincident appearance of anti-HIV CTL and control of initial acute HIV viraemia (Borrow et al., 1994). Also, the breadth of HIV Ag recognition by CTLs may determine the chronic viraemia set point after the acute viraemia stage of infection (Jones et al., 2004).

1.6.5 Human leukocyte antigen genotype associations to prognosis

The human major histocompatibility complex (MHC) is composed of genes located on the short arm of chromosome 6 [reviewed in (Janeway et al., 2001)]. The MHC class I proteins encoded in this complex are expressed on all nucleated cells and the class II proteins are expressed constitutively on APCs, T cells and endothelial cells when activated [reviewed in (Janeway et al., 2001)]. The MHC proteins express foreign and 'self' peptides that are recognised during infection and immune surveillance to discern between healthy and infected host cells. The most polymorphic region of the human genome is the MHC [reviewed in (Horton et al., 2004)], so some individuals may express HIV Ag via MHC class II more efficiently and control viral load better than others. Human leukocyte antigen (HLA) haplotypes DQ2-DR3-B8-Cw7-A1 and DQ1-DR1-B35-Cw4-A11 have been associated with rapid progression to AIDS (Just, 1995), whereas HLA-Bw4, B44 and B57 have been associated with long-term non-progression

to AIDS (Flores-Villanueva et al., 2001; Migueles et al., 2000), however the mechanism(s) that contribute(s) to the haplotype specific prognosis is still not known.

Homozygosity for any 2 Bw4 alleles is a marker for delayed AIDS progression. This raises the possibility that NK cells are involved in regulating AIDS progression since HLA-B molecules with the Bw4 motif serve as ligands for the natural killer cell receptor, killer immunoglobulin receptor (KIR) 3DL1 (Carrington and Bontrop, 2002). KIRs expressed on NK cells regulate inhibition and activation of NK cell responses through recognition of HLA class I molecules on the cell surface (Janeway et al., 2001).

1.6.6 T helper cells

There are two subsets of T helper cells (T_h cells): T_h1 and T_h2 . T_h1 cells stimulate the $CD8^+$ CTL, and macrophage ($M\phi$) cell-mediated immune response through secreted $IFN-\gamma$ and IL12 [reviewed in (Janeway et al., 2001)]. T_h2 cells stimulate (antibody) Ab production by B cells through secretion of IL4, IL6, and IL10 (the humoral response) [reviewed in (Janeway et al., 2001)]. The activation of a naïve T_h cell by a professional APC is via MHC class II interaction with the T_h cell receptor and CD4 [reviewed in (Janeway et al., 2001)]. Whether the T_h cell becomes type 1 or type 2 depends on the costimulation it receives at the point of Ag presentation [reviewed in (Janeway et al., 2001)].

Strong cell-mediated immunity is associated with low HIV viral loads (Clerici et al., 1996a; Clerici et al., 1996b; Clerici et al., 1993; Clerici et al., 1994; Clerici and Shearer, 1996; Kalams and Walker, 1998), and a strong T_h1 cell response is required to drive this response. Indeed, HIV-1-specific $CD4 T_h1$ cells expressing $IFN-\gamma$, combined with IgG2 antibodies are “predictors” of long-term non-progression to AIDS (Clerici et al., 1996a; Clerici et al., 1996b; Clerici et al., 1993; Clerici et al., 1994; Martinez et al., 2005).

The T_h1 and T_h2 response in HIV infection is skewed toward a T_h2 response in those individuals that progress to AIDS (Clerici et al., 1996b; Clerici et al., 1994; Clerici and Shearer, 1996). Cytokines of a type 2 profile (IL-4, 6, 10) are associated with the onset of disease, in addition to the inability of peripheral blood mononuclear cells (PBMC) taken from these patients to be unreactive to *in vitro* Influenza and HIV Env Ag

stimulation as measured by IL-2 production (Clerici et al., 1996b; Clerici et al., 1993; Clerici et al., 1994).

1.6.7 Humoral immunity

B cells produce Abs in response to stimulation by T_H2 cells [reviewed in (Janeway et al., 2001)]. If an Ab inhibits virus infection then it is considered neutralising: “the loss of infectivity which ensues when Ab molecule(s) bind to a virus particle, and usually occurs without the involvement of any other agency” (Dimmock, 1993). Neutralising antibodies (NAbs) probably work in concert with cell-mediated immunity to help control viral infection (Burton, 2002).

NAbs to HIV cannot be detected until after the initial peak viraemia, sometimes they do not appear until 17 months after acute infection (Aasa-Chapman et al., 2004), so they may play a role in control of the later stages of infection (Ferrantelli and Ruprecht, 2002). However, studies have suggested that complement (a cascade of proteins that act to form pores in and opsonize pathogens) in conjunction with non-NAbs directed against virus Env may help suppress initial peak HIV viraemia (Aasa-Chapman et al., 2005).

Seroconversion is the point at which anti-HIV-1 antibodies can be detected in the blood. HIV-1 infection is diagnosed by detecting antibodies specific to the virus which show-up 6-8 weeks after infection (Gurtler, 1996). This period is called “serological latency” and may be shorter than 6 weeks or several weeks longer, however inability to detect antibodies 3 months after infection is rare (Gurtler, 1996). Antibodies to Env, the immunodominant epitope of gp41 and Gag (p24 and p17) are detectable first. Following are antibodies to reverse transcriptase RT (p51 and p66) and integrase (IN) (p32). Antibodies to all of these HIV proteins persist for life (Gurtler, 1996).

1.7 Highly Active Anti-Retroviral Therapy: triple therapy.

The treatment of HIV infection with anti-retroviral drugs involves blocking viral replication [reviewed in (Dalglish and Weiss, 1999)]. The purpose of highly active anti-retroviral therapy (HAART) is to lower plasma viraemia to restore circulating

CD4⁺ T cell levels, thus the patient is able to fight opportunistic infection and live longer [reviewed in (Back et al., 2002)]. The first of these drugs to be used was Zidovudine [Azidothymidine(AZT)], which was licensed for symptomatic disease in 1987 [(Brook, 1987; Fischl et al., 1987; Parks et al., 1988; Yarchoan et al., 1986; Yarchoan et al., 1988) reviewed in (Dalglish and Weiss, 1999)].

1.7.1 Nucleoside reverse transcriptase inhibitors

NRTIs like AZT, lamivudine (3TC), didanosine (ddI), zalcitabine (ddC) and stavudine (d4T) are converted to their active triphosphate forms within cells [reviewed in (Back et al., 2002)]. The mode of anti-retroviral action is to act as viral DNA chain terminators as the proviral genome is reverse transcribed from the viral RNA [reviewed in (Hightower and Kallas, 2003)].

1.7.2 Non-nucleoside reverse transcriptase inhibitors

The non-nucleoside reverse transcriptase inhibitors (NNRTIs), Nevirapine and Efavirenz have an aromatic structure and bind to a hydrophobic pocket near the polymerase active site [reviewed in (Hightower and Kallas, 2003)]. Unlike HIV-1, these drugs do not affect the *in vitro* replication of HIV-2 [reviewed in (Hightower and Kallas, 2003)]. It is thought that this natural resistance is conferred by amino acid Leu-188 that is in close proximity to one of the two hydrophobic pockets of RT [reviewed in (Hightower and Kallas, 2003)].

1.7.3 Protease inhibitors

The HIV-1, HIV-2, and SIV proteases belong to the aspartyl-protease family. They are required to post-translationally cleave polyproteins into structural proteins and functional enzymes required for infectivity [reviewed in (Hightower and Kallas, 2003)]. Protease inhibitors such as Indinavir, Saquinavir, Nelfinavir, and Ritonavir compete for the protease active site and have been shown to bind HIV-1 protease with 10 to 100 times greater affinity than the HIV-2 protease (Tomasselli et al., 1990), and are therefore much more effective against HIV-1 than HIV-2 infection.

1.7.4 Fusion inhibitor

Enfuvirtide is a 36 amino acid peptide specific for a portion of the gp41 HR2 region of the HIV-1 laboratory strain LAI but is ineffective at inhibiting HIV-2 or SIV replication [reviewed in (Greenberg et al., 2004)], it is also effective at inhibiting HIV-1 group O replication (Poveda et al., 2005). Enfuvirtide binds to the six-helix bundle conformation, of the gp41 portion of the Env protein, which forms during the fusion process [reviewed in (Greenberg et al., 2004)]. Fusion is therefore blocked mid process and the virion remains tethered to the cell via the fusion peptide.

1.7.5 Integrase inhibitors

The β -diketo acids have been described as potent inhibitors of HIV IN activity (Hazuda et al., 2000). Their specific action is to inhibit strand transfer (3' end-joining step) during the integration reaction (fig 1.7)(Marchand et al., 2002). The β -diketo acids are specific for the strand transfer reaction in the nanomolar range, do not affect 3' processing in treated cells, so they were 'proof of concept' that inhibition of strand transfer activity equates to antiviral activity [reviewed in (Pommier et al., 2005)]. A diketo acid is in phase I clinical trials as of March 2005 [reviewed in (Pommier et al., 2005)].

1.7.6 Small molecule receptor-binding inhibitors

A small molecule HIV replication inhibitor has been reported that blocks the binding of HIV to its primary CD4 receptor and is a promising candidate for clinical trials (Lin et al., 2003; McKnight and Weiss, 2003). However, coreceptor and receptor blocking drugs are currently unavailable for treatment of HIV infection [reviewed in (Pommier et al., 2005)].

1.7.7 Drug resistance

The list of amino acids associated with HIV-1 drug resistance is too long to discuss here but there are some interesting comparisons between HIV-1 and HIV-2. Some HIV-1 resistance mutations occur naturally in HIV-2, such as Leu-188, as described earlier in 'Non-nucleoside reverse transcriptase inhibitors' section. The RT error rate, diploid

RNA genome recombination, and the high rate of HIV replication all contribute to the generation of resistance mutations to anti-retroviral drugs (Mansky and Temin, 1995; Rezende and Prasad, 2004). Drug resistance has been mapped to numerous mutations in the HIV genome for each of the categories of antiretroviral drugs currently in use [reviewed in (Back et al., 2002)], even for the IN inhibitors that have yet to see widespread use [reviewed in (Pommier et al., 2005)].

1.8 The retroviruses and their phylogeny

Retroviruses are defined by the reverse transcription of their RNA genome into DNA for integration into the host genome. They carry a diploid RNA genome in the virion that is of positive polarity. The spumaviruses, however, carry a partially and completely reverse transcribed DNA genome in the particle that was formed in the producer cell (Delelis et al., 2003; Moebes et al., 1997; Yu et al., 1999).

HIV-1 and HIV-2 are “complex” retroviruses in the family *Retroviridae* of the genus *lentivirus*. MLV is a “simple” retrovirus in the gamma retrovirus genus. In the *Retroviridae*, the simple retroviruses are divided into the alpha, beta, and gamma retroviruses (fig 1.2). The delta and epsilon retroviruses, lentiviruses and spumaviruses make up the complex retroviruses (fig 1.2) [reviewed in (Coffin et al., 1997)]. There are four human retroviruses, and they all cause disease in humans: HTLV-1, HTLV-2, HIV-1, HIV-2; and the simian foamy viruses (SFVs). HTLV-1 infection is associated with tropical spastic paraparesis (TSP) or HTLV-1 associated myelopathy (HAM), and adult T cell leukaemia (ATL) (Gallo et al., 1981; Gessain et al., 1985; Hinuma et al., 1981; Miyoshi et al., 1981; Poiesz et al., 1980; Roman et al., 1987). The pathogenic role of HTLV-2 remains unclear except in the presence of rare cases of CD8 lymphoproliferative disorders and neuromyelopathies (Fouchard et al., 1995; Hall et al., 1996). HIV-1 and HIV-2 both cause AIDS in infected individuals. The SFVs don't cause any known disease because animal keepers who seroconvert to SFV as a result of being bitten or scratched by a range of Old World primates, and a laboratory worker accidentally infected with SFV, have all remained healthy for over a decade (Heneine et al., 1998). However the first human foamy virus (HFV) isolate to be described was

isolated from nasopharyngeal carcinoma tissue from a Kenyan patient (Achong et al., 1971; Epstein et al., 1974).

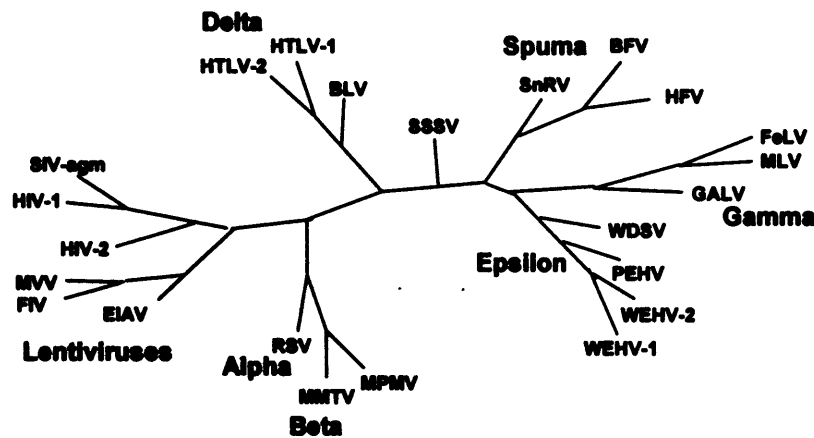


Figure 1.2. Unrooted phylogenetic tree of the *Retroviridae*.

SIV-agm = simian immunodeficiency virus isolated from African green monkeys, MVV = Maedi-Visna virus, FIV = feline immunodeficiency virus, EIAV = equine infectious anaemia virus, RSV = Rous sarcoma virus, MMTV = mouse mammary tumour virus, MPMV = Mason-Pfizer monkey virus, WEHV-1 and 2 = walleye epidermal hyperplasia viruses 1 and 2, PEHV = Perch epidermal hyperplasia virus, WDSV = walleye dermal sarcoma virus, GALV = gibbon ape leukaemia virus, MLV = murine leukaemia virus, FeLV = feline leukaemia virus, HFV = human foamy virus, BFV = bovine foamy virus, SnRV = snakehead retrovirus, SSSV = salmon swim bladder sarcoma virus, BLV = bovine leukaemia virus, HTLV-1 and 2 = human T cell leukaemia virus 1 and 2. Adapted from (Knipe et al., 2001).

1.8.1 Complex versus simple

The complex retroviruses, unlike the simple retroviruses, have accessory genes. The simple retroviruses encode only the Gag, Pro, Pol, and Env gene products, with some exceptions (see below). The complex retroviruses encode all of these products in addition to accessory genes that have a range of functions as described for HIV in *The HIV life cycle* section [reviewed in (Knipe et al., 2001)]. The genome structures of complex retroviruses HIV-1 and HIV-2 along-side the simple retrovirus MLV are shown in fig 1.3.

1.8.2 Genome structure

The alpha, beta and gamma retroviruses have “simple lifestyles,” as introduced above (Coffin et al., 1997). Simple retroviruses do not encode additional proteins that directly and specifically affect viral RNA synthesis or processing. This simple pattern of RNA regulation is correlated with the simple pattern of mRNA splicing. Most of the simple retroviruses make only one spliced *env* mRNA, none of the simple retroviruses make multiply spliced mRNAs as is the case with MLV in fig 1.3, below.

MMTV, a member of the beta retrovirus genus (fig 1.2), encodes a superantigen (*sag*) that overlaps U3 of the LTR and this adds another spliced mRNA in addition to the spliced *env* transcript. Though several members of this genus are associated with neoplastic disease none encode a known oncogene.

Complex retroviruses like the delta retroviruses, lentiviruses and spumaviruses have multiple splice donor sites in their genomes which give rise to complex patterns of mRNA (fig 1.10), a greater variety of gene products and the encoding of viral proteins in all three reading frames, like HIV-1 and HIV-2 in fig 1.3.

The epsilon retroviruses have singly spliced genomes to accommodate translation of *env* but are complex in that they encode from one to three open reading frames (ORF). ORFa, present in all three walleye retroviruses is a viral homologue of cyclin D.

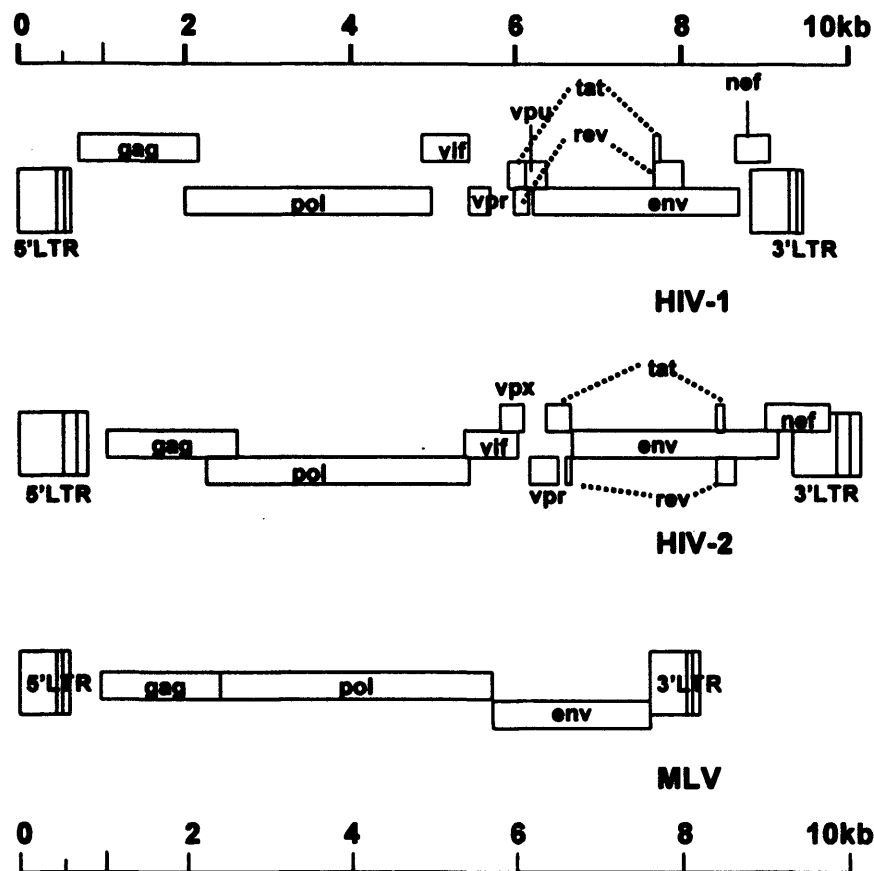


Figure 1.3. Comparison of the genome structure of complex retroviruses HIV-1 and HIV-2, and the simple retrovirus MLV.

The *gag* gene encodes for the principle structural protein **group specific antigen (Gag)**, is packaged as a full-length protein or as a fusion with Pol (Gag-Pol). After virion budding from the producer cell Gag is cleaved by the viral protease into HIV-1 and HIV-2 matrix (MA), capsid (CA), nucleocapsid (NC), spacer peptide 1 (SP1), spacer peptide 2 (SP2) and P6. MLV Gag is also cleaved post-budding into MA, CA, NC, and P12. The *pol* gene encodes for **Polymerase (Pol)** that is also cleaved by the viral Protease into **Reverse transcriptase (RT)**, **Integrase (IN)** and **Protease (PR)**, required for reverse transcribing the virus RNA genome into DNA (RT), integrating the viral cDNA genome into the host DNA (IN) and cleavage (PR) and maturation of virus proteins (PR). Pol is not expressed as efficiently as Gag and is expressed as a fusion with Gag (Gag-Pol) by -1 ribosomal frame-shifting (HIV) during *gag* translation or termination suppression (MLV). All retroviruses contain 5' and 3' long terminal repeats (LTRs) that contain promoter, polyadenylation and transcriptional start sequences. The *env* gene encodes for the **Envelope (Env) surface gp120 (SU) and gp 41 (TM) subunits of HIV-1 and gp105 (SU) and gp36 (TM) of HIV-2**. The MLV Env is also expressed from a spliced mRNA and produces a glycosylated precursor, **gPr80 Env** that is cleaved by a cellular signal peptidase and cellular furin to produce the mature SU and TM proteins. SU (gp70) is glycosylated but TM is not and the two are non-covalently linked by disulfide bridge(s) (Opstelten et al., 1998). TM p15E is further cleaved by MLV PR to produce p12E

and p2E (the R peptide) (Green et al., 1981). This processing is required for fusion of the virion membrane and the host-cell plasma membrane, mediated by Env, during infection. Viral infectivity factor (Vif), viral protein R (Vpr), viral protein X (Vpx), viral protein U (Vpu), negative infectivity factor (Nef), transactivator of transcription (Tat), regulator of expression of viral proteins (Rev).

1.8.3 Epidemiology and classification of the primate lentiviruses

The HIVs and SIVs are currently classified into six groups of primate lentiviruses (table 1.1) (Barlow et al., 2003; Hahn et al., 2000), and additional groups will probably emerge as more full-length sequences are elucidated from different primate species (Peeters et al., 2002). There are at least 18 distinct lentiviruses that naturally infect African primate species (Hahn et al., 2000) and over 40 different SIVs have been described from their natural or heterologous species (Apetrei et al., 2004).

Table 1.1: The six groups of primate lentiviruses.

Group	Virus	Host species
1	HIV-1 (Barre-Sinoussi et al., 1983)	<i>Homo sapiens</i> Human
	SIVcpz (Peeters et al., 1992; Peeters et al., 1989)	<i>Pan troglodytes</i> Chimpanzee
2	HIV-2(Clavel et al., 1986a),	<i>Homo sapien</i> Human
	SIV _{SMM} (Murphey-Corb et al., 1986),	<i>Cercocebus atys</i> Sooty mangabey,
	SIVmac (Daniel et al., 1985)	<i>Macaca spp</i> Macaques
3	SIVagm (Ohta et al., 1988)	<i>Cercopithecus spp</i> African green monkeys
4	SIVmnd (Tsujimoto et al., 1988)	<i>Papio sphinx</i> Mandrill
5	SIVsyk (Emau et al., 1991)	<i>Cercopithecus albogaris</i> Sykes monkey
6	SIVcol (Courgnaud et al., 2001)	<i>Colobus Guerza</i> Colobus monkeys

HIV-1 consists of groups M (main), N (new), and O (outlier). Group M is responsible for the current pandemic and has been further subdivided into 11 clades, A through K. Subtype C predominates globally with 47% of new infections in the year 2000, clade A accounted for 30% (including AE and AG), circulating recombinant forms (CRFs) accounted for 18%, and clade B accounted for 12% of all new infections in 2000 (Julg and Goebel, 2005). Clade B is found in North America, South America, Europe and Southeast Asia. Clade C predominates in sub-Saharan Africa and Southeast Asia with BF, AE, BC, AG, and AB CRFs in South America, southeast Asia, China, Eastern Europe and central Africa, respectively [<http://www.iavireport.org/issues/0803/images/HIV-clademap.pdf>, accessed October, 2005; (Julg and Goebel, 2005)].

HIV-2 is categorised into 7 subtypes, A through G. Subtypes A and B cause AIDS and have been isolated from individuals as infectious virus whereas only partial nucleotide sequences have been obtained for subtypes C through G (Gao et al., 1994). HIV-2 is found primarily in West Africa; subtype A is the most prevalent and found in The Democratic Republic of the Congo, Mali, Gambia, Cape Verde Islands, Guinea Bissau, Ghana, Senegal, and Liberia whereas subtype B is found almost exclusively in the Ivory Coast and Ghana (Ellenberger et al., 1999; Ishikawa et al., 2001; Peeters et al., 2003; Pieniazek et al., 1999). In contrast to HIV-1, the HIV-2 epidemic is waning with a gradual decrease in the trend of HIV-2 seroprevalence (Bouckennooghe and Shandera, 1999).

1.8.4 The origins of HIV-1 and HIV-2

Geographical location and sequence data suggest that HIV-2 was derived from SIV of Sooty Mangabeys, SIV_{SMM}. The presence of HIV-2 was confirmed through molecular characterisation (Clavel et al., 1986a), approximately one year after serological screening suggested its presence (Barin et al., 1985). HIV-2 and SIV_{SMM} share identical genome structure (Hirsch et al., 1989), they do not separate into distinct phylogenetic lineages based on their species of origin (table 1.1) (Hahn et al., 2000), and there is “geographic coincidence” between the natural habitat of the Sooty

Mangabey and where HIV-2 is endemic (Chen et al., 1996b; Hahn et al., 2000). Each of the seven subtypes of HIV-2 are as divergent in sequence as the SIV_{SMM} sequences are from each other, in the same geographic location, which supports the hypothesis that each HIV-2 subtype is the result of an independent zoonotic event of Sooty Mangabey to Human (Apetrei and Marx, 2004; Apetrei et al., 2004; Chen et al., 1997; Chen et al., 1996b; Gao et al., 1994; Gao et al., 1992; Hahn et al., 2000).

HIV-1 is similar to SIV from chimpanzees. However, the origin of HIV-1 was more difficult to elucidate than that of HIV-2 (table 1) (Hahn et al., 2000). At one point, there were only two full-length sequences of SIV_{CPZ}: SIV_{CPZ-GAB1}, SIV_{CPZ-ANT} and a partial Pol sequence named SIV_{CPZ-GAB2} (Vanden Haesevelde et al., 1996), which did not provide a clear epidemiological SIV_{CPZ} to HIV-1 link. A clearer association between HIV-1 and SIV_{CPZ} was not made until additional SIV_{CPZ} sequences were obtained [(Bibollet-Ruche et al., 2004; Gao et al., 1999; Rambaut et al., 2001; Switzer et al., 2005; Worobey et al., 2004) reviewed in (Hahn et al., 2000)]. The phylogenetic starburst of group M HIV-1 sequences and the interspersed SIV_{CPZ} sequences between HIV-1 groups M, N and O suggest that these three HIV-1 groups are derived from three independent zoonotic transfers of virus from chimpanzee to human (Bibollet-Ruche et al., 2004; Gao et al., 1999; Hahn et al., 2000; Rambaut et al., 2001; Worobey et al., 2004), so all of the circulating subtypes of group M were derived from one zoonotic event. The circulating subtypes of HIV-2 however were each thought to have been derived from separate zoonotic events [reviewed in (Hahn et al., 2000)]. It is deduced that the current (December, 2004) 39 million HIV-1 group M infections worldwide are the result of 1 zoonotic transfer of SIV_{CPZ} to a human.

1.9 Cellular tropism and receptors of HIV

The cell tropism of HIV is best explained in the context of receptor and, most importantly, coreceptor usage. Therefore the receptor and coreceptors of HIV will be discussed before the cellular tropism of HIV.

1.9.1 The HIV receptor

It has been shown that CD4 is the primary receptor for HIV-1 (Clapham PR, 2001; Clapham and McKnight, 2002; Dalgleish et al., 1984; Klatzmann et al., 1984). Anti-CD4 mabs and soluble recombinant CD4 (sCD4) can block infection of HIV-1, HIV-2 and SIV viruses (Clapham et al., 1989; Sattentau et al., 1988; Weiss et al., 1988), suggesting that CD4 is the main receptor for all of the primate lentiviruses.

1.9.2 Coreceptors

It was observed two years after the discovery of the CD4 receptor that additional cofactors were required for HIV entry and infection. It was shown that HIV-1 could bind murine cells and non-permissive human cells expressing CD4, but could not fuse and enter (Harrington and Geballe, 1993; Maddon et al., 1986). However, HeLa/non-permissive cell fusions were permissive to infection suggesting the presence of an entry cofactor (Harrington and Geballe, 1993; Maddon et al., 1986). Fusin (CXCR4) was the first identified coreceptor required for HIV infection of SI isolates (Feng et al., 1996), and discovery of the R5 coreceptor (Dragic et al., 1996), required for MØ tropism (Alkhatib et al., 1996), followed (table 1.2). Also, the discovery of dual coreceptor and broad coreceptor using isolates helped to explain dual MØ and T cell tropic isolates (table 1.2) (Berson et al., 1996; Doranz et al., 1996).

All of the HIV coreceptors are members of the 7-TM spanning receptor superfamily. These receptor proteins signal through the heterotrimeric G-proteins upon chemokine ligation.

HIV-2 has been shown to use a broader range of coreceptors than HIV-1 *in vitro* (Guillon et al., 1998; McKnight et al., 1998; Morner et al., 1999; Morner et al., 2002; Owen et al., 1998), but this does not correlate with HIV-2 pathogenicity *in vivo* (Blaak et al., 2005; Morner et al., 1999; Morner et al., 2002; van Der Ende et al., 2000). A comprehensive list of HIV-1 and HIV-2 coreceptors is listed in table 1.2. The HIV coreceptors and associations with cell tropism are discussed in more detail in chapter 3.

Table 1.2: Coreceptors used by HIV-1, HIV-2, and SIV.

Coreceptor	Virus	Cell/tissue type	Reference
CCR1	HIV-2	Activated T cells, mono, DC	(Guillon et al., 1998; McKnight et al., 1998)
CCR2b	HIV-1, HIV-2, SIV	Mono, T cells	(Chen et al., 1998; Doranz et al., 1996)
CCR3	HIV-1, HIV-2	Eosinophils, microglia, T _H 2	(Choe et al., 1996; Sol et al., 1997)
CCR4	HIV-2	T _H 2,	(McKnight et al., 1998; Owen et al., 1998)
CCR5 (R5)	HIV-1, HIV-2, SIV	Activated T cells, mono, DC	(Deng et al., 1996; Sol et al., 1997)
CCR8	HIV-1, HIV-2, SIV	T _H 2, brain, thymocytes	(Rucker et al., 1997)
CCR9	HIV-1	Lymphocytes, brain, placenta	(Choe et al., 1998)
CXCR2	HIV-2	Neutrophils, brain	(Bron et al., 1997)
CXCR4 (X4)	HIV-1, HIV-2, SIV, FIV	Lymphocytes, mono, brain, progenitor cells	(Feng et al., 1996; Schols and De Clercq, 1998; Willett et al., 1997)
CXCR5	HIV-2	B cells	(Kanbe et al., 1999)
CX ₃ CR1	HIV-1, HIV-2	NK cells, CD8 T cells, brain	(Reeves et al., 1997; Rucker et al., 1997)
GPR1	HIV-1, HIV-2, SIV	MØ, brain	(Farzan et al., 1997; Shimizu et al., 1999)
GPR15 (BOB)	HIV-1, HIV-2, SIV	T cells, colon	(Deng et al., 1997; Farzan et al., 1997)
STRL33 (Bonzo)	HIV-1, HIV-2, SIV	T cells, mono, placenta	(Deng et al., 1997; Liao et al., 1997)
APJ	HIV-1, HIV-2	CNS	(Choe et al., 1998;

	SIV		Edinger et al., 1998)
ChemR23	HIV-1, SIV	MØ, DC	(Samson et al., 1998)
RDC1	HIV-1, HIV-2, SIV	Lymphocytes, brain	(Shimizu et al., 2000)
Leukotriene receptor (BLTR)	B4 HIV-1	Leukocytes	(Owman et al., 1998)
US28 _{CMV}	HIV-1, HIV-2	CMV-infected cells	(Pleskoff et al., 1997)
D6	HIV-1, HIV-2	MØ, PBMC, brain	(Neil et al., 2005)

1.9.3 Attachment receptors

Depending on the route of transmission, there are several factors that affect virion-cell binding, such as extracellular fluid (Maher et al., 2005), and host cell surface attachment proteins and proteoglycans (Ugolini et al., 1999).

Abs directed against the cell-cell attachment protein, ICAM-3 have been shown to inhibit HIV infection (Sommerfelt and Asjo, 1995) and it was once thought that LFA-1 was required for HIV fusion because mabs to LFA-1 could block syncytium formation (cytopathic cell fusion effect of HIV in culture) (Hildreth and Orentas, 1989; Ugolini et al., 1999). Intercellular attachment proteins already present on the surface of producer cells may bud off with virions so incorporation of these cellular proteins on the virion may be a non-selective/random process (Tremblay et al., 1998). ICAMs on the surface of virions may aid virion-target cell attachment by interaction with the associated ICAM receptor and enhance infectivity (Fortin et al., 1997), and can even make the virus refractory to Ab neutralisation (Fortin et al., 2000).

Heparin sulphate proteoglycans (HSPG) are expressed to high levels on epithelial and endothelial cells attracting engagement of HIV virion with their net anionic charge (Ugolini et al., 1999), thus supporting virus absorption to the cell surface. The preferred binding of X4 viruses with HSPG is associated with the higher V3 loop charge compared to R5 viruses (HIV V3 loops are explained in greater detail in chapter 3) (Ohshiro et al., 1996; Ugolini et al., 1999).

The binding of virus by dendritic cells (DCs) at sites of virus exposure (mucosal tissue) may lead to unwitting presentation of infectious virus to target T cells in lymph nodes (van Kooyk and Geijtenbeek, 2003). The DC C-type lectin, specific intercellular adhesion molecule-grabbing nonintegrin (DC-SIGN) (Geijtenbeek et al., 2000), and the endothelial cell expressed DC-SIGNR (Pohlmann et al., 2001), have been shown to bind HIV (or any particles with high mannose sugars on glycoproteins) and promote internalisation/protection of virus by DCs, until presentation via the immunological synapse to the unsuspecting T cell. The DC-SIGNs are internalisation receptors that internalise with bound ligand (van Kooyk and Geijtenbeek, 2003) and therefore promote internalisation and protection of virus by DCs, allowing virion to remain infectious for prolonged periods (van Kooyk and Geijtenbeek, 2003).

Galactosyl ceramide and its derivatives (sulphated) have been implicated as alternative receptors for HIV entry (Bhat et al., 1991; Harouse et al., 1991), although at much lower efficiency than via preferred receptor and coreceptor. The bond between the galactose sugar residue and the ceramide-lipid backbone is required for binding to HIV gp120 Env protein (Bhat et al., 1991). This glycolipid may be used by HIV to infect CD4⁽⁺⁾ colon epithelial cells in the gastrointestinal tract (Yahi et al., 1992), and may be of particular importance because anti-CD4 Ab doesn't inhibit HIV infection of human colon epithelial cells (Fantini et al., 1991). However given how inefficient these processes are *in vitro* it is difficult to envisage a major *in-vivo* role.

1.9.4 Cells infected: transmission through to onset of AIDS

HIV preferentially infects haematopoietic cells that express CD4 and the relevant 7-TM coreceptor(s), namely; DCs, MØ and T cells (Clapham and McKnight, 2002). During transmission of HIV, immune cells of the mucosae are infected and those cells that express the pertinent receptor and coreceptor are the targets of attachment and infection (Clapham and McKnight, 2002; de Roda Husman and Schuitemaker, 1998; Moore et al., 2004). The most relevant cells infected, during transmission, *in-vivo* are the R5 expressing cells such as resident MØs, T cells and DCs (Clapham and McKnight, 2002; Davis and Doms, 2004; de Roda Husman and Schuitemaker, 1998; Miller and Shattock, 2003; Moore et al., 2004; Schuitemaker, 1994; Wilflingseder et al., 2005).

A model of transmission that has gained acceptance is the one where DCs [e.g. Langerhans cells (LCs)] and MØs present in the mucosae become productively infected (via CD4 receptor and R5 coreceptor) or transport virus particles via C-type lectins (e.g. DC-SIGN). These HIV containing “Trojan horses” then migrate to draining lymph nodes, producing/presenting HIV for subsequent infection of susceptible T cells (Clapham and McKnight, 2002; Davis and Doms, 2004; Moore et al., 2004; Wilflingseder et al., 2005). Activated CD4⁺ T cells are present in ample numbers in inflamed mucosae during ulcerative genital diseases so these cells are also a target for HIV infection during transmission (Ho et al., 1995).

Follicular dendritic cells (FDCs) are not infected by HIV but enhance cell-free virus infection by promoting the upregulation of X4 on surrounding T cells *In vitro* (Estes et al., 2002; Taruishi et al., 2004). FDCs, present in the lymph nodes, may create a more suitable microenvironment for virus transmission in the host.

X4 strains emerge in 40-50% of subtype B infections (Connor et al., 1997), and X4 viruses in symptomatic individuals demonstrate a broader tropism for different T cell subsets (Clapham and McKnight, 2002). A nomenclature system has been derived for HIV viruses where those that use CCR5 as a coreceptor for entry are termed R5, those that use CXCR4 are termed X4 and those that can use both coreceptors equivalently for entry are R5X4 viruses (Berger et al., 1998).

In T cells, R5 expression is found primarily on memory T cells whereas X4 is more widespread and is expressed to higher levels on naïve T cells (Bleul et al., 1997). Accordingly, in the blood of individuals carrying R5 viruses the CD4⁺CD45RO⁺ memory T cells carry most of the proviral load, with a minority of CD4⁺CD45RA⁺ naïve T cells infected, and when X4 viruses emerge a greater proportion of CD4⁺CD45RA⁺ naïve T cells become infected (Blaak et al., 2000; Clapham and McKnight, 2002; Ostrowski et al., 1999). Also, with regards to cells in general, X4 is more widely expressed than R5, which would lend a broader cell tropism to X4 using HIV viruses than R5 viruses (Moore et al., 2004). The infection of MØs by HIV and the role that these cells play in infection is discussed in more detail in chapter 3.

1.10 HIV Vaccines and microbicides

There is currently no prospect for an efficacious vaccine against HIV-1 infection (Desrosiers, 2004; Letvin, 2005). Nothing tried thus far has provided therapeutic assistance to help fight infection let alone sterilising immunity (Desrosiers, 2004; Letvin, 2005; McMichael and Hanke, 2003). Therefore there is more research than ever on topical microbicides and chemical prophylaxis.

1.10.1 Vaccines

The results of the first phase 3 HIV-1 vaccine trials were released in 2003. The Vaxgen gp120 trials were considered a “failure” because they did not provide protection against infection and did not lower viral loads (Desrosiers, 2004). It is becoming increasingly apparent that traditional vaccine approaches will not work to vaccinate against HIV and more novel strategies are starting to be adopted (Desrosiers, 2004; Letvin, 2005; McMichael and Hanke, 2003). As of March 2004, there were 13 different products at various stages of clinical testing in more than 20 individual human trials (Desrosiers, 2004).

1.10.2 Microbicides

Microbicides are topical formulations designed to block HIV-1 infection when applied to genital mucosal surfaces before sexual intercourse.

A surfactant called nonoxynol-9, known to non-specifically disrupt enveloped viral particles, was shown in clinical trials to destroy membranes of epithelial cells lining mucosal surfaces (Shattock and Moore, 2003). As a result, nonoxynol-9 did not protect against HIV infection but increased the chances of becoming infected (Shattock and Moore, 2003). Therefore any potential microbicide must not reduce natural defences against HIV infection.

The most appealing microbicide candidates will specifically target the HIV virus, such as: antibodies, proteins and small molecule inhibitors that bind the viral Env glycoproteins gp 120 and gp 41 or glycan residues present on the surface of the viral

Env. These will ideally inactivate the virus before it has the chance to infect (Shattock and Moore, 2003).

Chemical approaches are also being pursued in light of promising pre-clinical results. (R)-9-(2-phosphonylmethoxypropyl) adenine (PMPA) has proven effective at preventing SIV infection of macaques when delivered subcutaneously, once daily, as pre- and post-exposure prophylaxis (Tsai et al., 1995). An RT inhibitor, with few side effects, tenofovir disoproxil fumarate (TDF) is currently in clinical trials to determine its efficacy as preexposure prophylaxis (Grant et al., 2005).

1.11 The HIV infection cycle

'The HIV infection cycle' section of this thesis describes the viral and cellular proteins when their action becomes relevant to the retroviral replication cycle. The pertinent viral and cellular proteins are discussed in detail in the subsection where they have been highlighted in **bold**. Therefore there is not a specific section in this thesis that lists the HIV gene products and their multitude of functions.

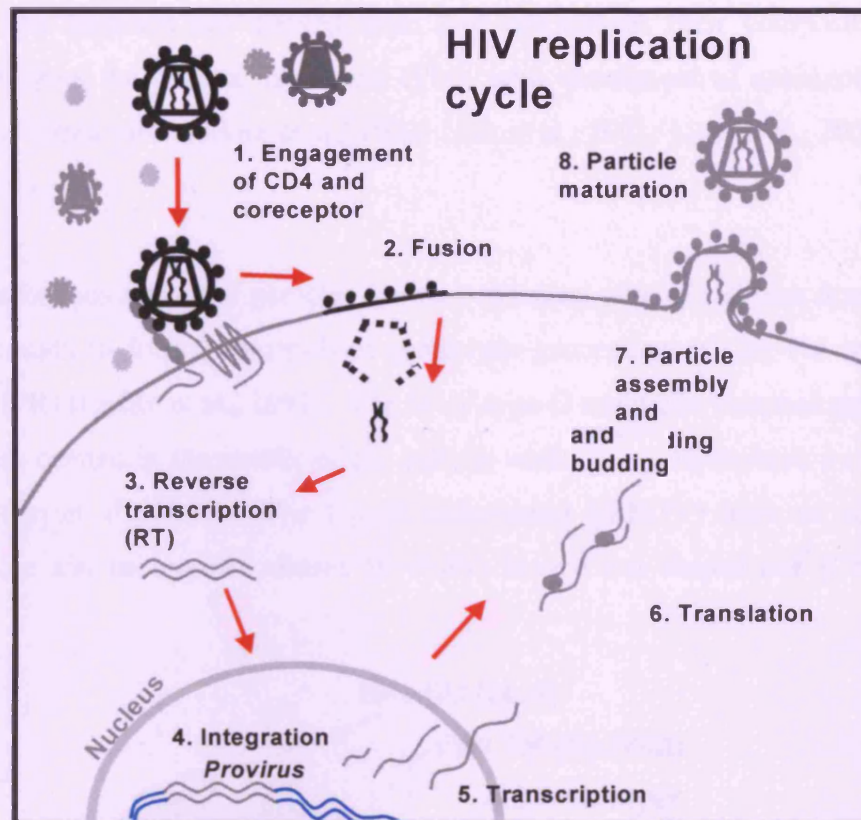


Figure 1.4. HIV replication cycle.

Step 1: virion engages CD4 receptor and 7-TM coreceptor. **Step 2:** conformational change caused by coreceptor engagement initiates fusion and mixing of virus and cell membranes, resulting in the delivery of the virus contents into the cell. **Step 3:** reverse transcription of the virus RNA into DNA. **Step 4:** integration of the viral DNA provirus into the host genome. Host genomic DNA is shown in blue and the integrated proviral cDNA is shown in grey. **Step 5:** the integrated provirus is transcribed by RNA polymerase II to produce spliced and unspliced RNA species. **Step 6 and 7:** the transcribed RNA is translated and viral proteins and RNA are assembled into immature virion that buds from host cell membranes. **Step 8:** maturation of the budded particle by virus encoded PR that results in formation of the conical shaped capsid that is typical of lentiviruses.

1.11.1 The infectious retrovirus particle

Like many other types of viruses not all retroviral virions are infectious. Typically the ratio of physical to infectious particles can be from 100:1 to 10 000:1 or greater (for a comparison of physical to infectious particle ratio between HIV-1 and HIV-2 see fig 3.5 in chapter 3). Retroviral particles are composed of, typically, 1-2% RNA, 35% lipids, and 60-65% proteins (Aloia et al., 1993; Coffin et al., 1997; Quigley et al., 1971), and

although the particles are derived from host membranes their composition differs somewhat from the plasma membrane (PM), with enrichment of sphingomyelin and cholesterol (lipid rafts) (Aloia et al., 1993; Liao et al., 2001; Liao et al., 2003; Nguyen et al., 2003).

Mature infectious retroviral particles have a condensed core of electron dense material that is thought to form as a result of proteolytic processing of Gag-Pol by the virus protease (PR) (Coffin et al., 1997). The MLV type-C retrovirus morphology is a dense round core centred in the middle of the particle while Lentiviruses have a cone shaped core (Coffin et al., 1997). The type-B retroviruses (MMTV) have an eccentrically placed core and the type-D viruses (M-PMV) have a rod shaped one (Coffin et al., 1997).

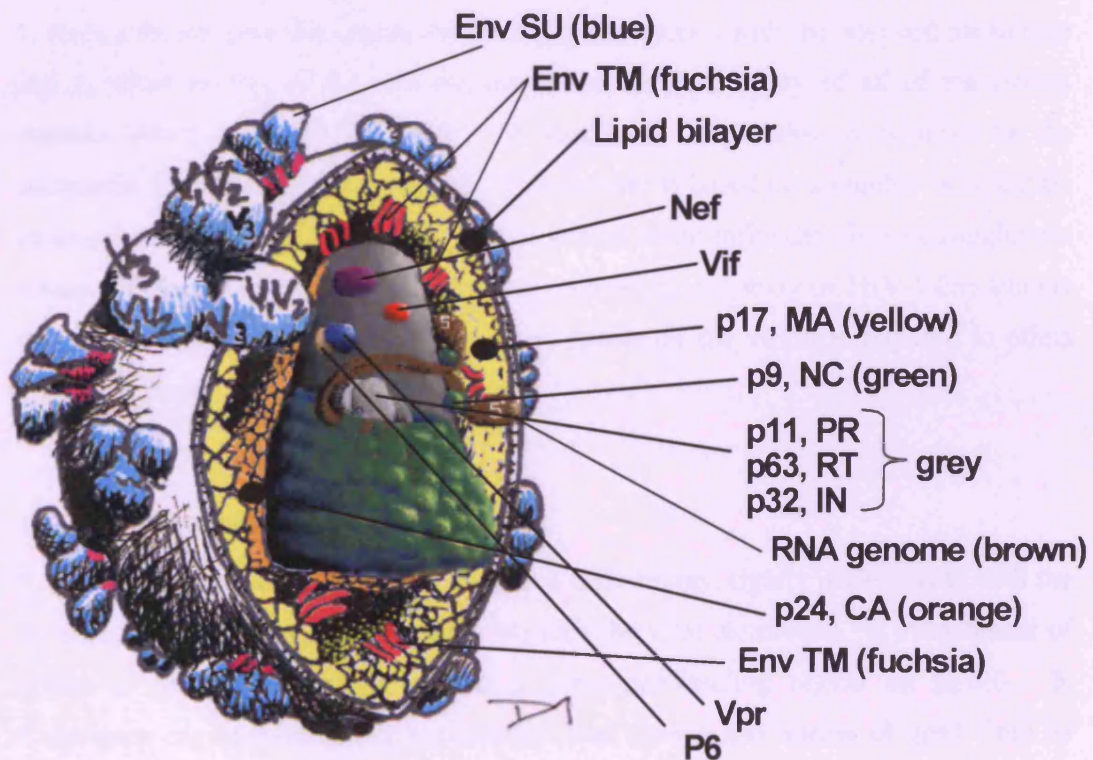


Figure 1.5. Composition of the HIV-1 virion (authors rendition).

Conformation of V1-V3 loops within trimeric Env was obtained from (Chen et al., 2005). The interaction of Env TM gp41 (fuchsia) with MA is derived from molecular modelling in (Forster et al., 2000). The dimeric RNA genome (brown) is shown coated in nucleocapsid (NC, green), spilling out of the orange capsid core, with the 5' ends exposed.

1.11.2 Receptor engagement

Initial HIV-receptor interaction during successful virus entry occurs when CD4 binds the CD4 binding site of the Env gp120 (SU) subunit (figs 1.4 and 1.5). CD4 receptor binding induces a conformational change in Env that allows a positively charged region near the bridging sheet that lies at the base of the V1V2 and V3 loops, to interact with the coreceptor (Doms, 2000; Rizzuto and Sodroski, 2000; Rizzuto et al., 1998). Successful coreceptor binding results in conformational rearrangement of gp41 (TM) (fig 1.5), and fusion (fig 1.4).

1.11.3 Fusion

The fusion process is mediated by the gp41 subunit of Env. The purpose of fusion is to 1, force a fusion pore that makes the viral Env continuous with the host cell membrane and 2, allow mixing of the two membranes to enable delivery of all of the virions contents into the cell (Doms, 2000). At least six R5 molecules are required for the successful formation of a fusion pore. This number is based on a number of scientific assumptions but is compatible with what is known about influenza virus hemagglutinin (Doms, 2000; Ellens et al., 1990). However, a more recent study on HIV-1 Env trimers themselves has suggested that just one Env trimer on the virion is required to effect virus-host fusion (Yang et al., 2005).

1.11.4 Fusion mechanism

1, Prior to fusion each gp41 molecule is in a high energy, tightly packed state with the N-terminal fusion peptide folded back towards the viral membrane. 2, Attachment of gp120 to the CD4 receptor exposes a coreceptor-binding region on gp120. 3, Coreceptor engagement causes a conformational change and release of gp41 from its high-energy state. 4, The fusion peptide extends out towards, and inserts into, the target cell membrane. 5, The HR1 and HR2 regions of gp41 are in consecutive alignment at this stage and they then 'fold in' to meet each other, which results in the formation of the six-helix bundle. The purpose of the formation of the six-helix bundle is to bring

the host cell and virus membranes into 6, close apposition to promote virus-cell membrane fusion and formation of the fusion pore.

1.11.5 Entry

The term 'entry' is used here to describe the topographical region of the cell where fusion takes place. This is especially relevant to chapter 4 of this thesis, which deals with endocytosis of virus particles and the resulting restriction of HIV-1 and HIV-2.

Several endocytic pathways have been implicated in the productive entry of viruses into the host cell (Marsh and Pelchen-Matthews, 2000; Sieczkarski and Whittaker, 2002a). One of the first viruses shown to utilise the clathrin endocytic route was Semliki forest virus (Helenius et al., 1980), also demonstrated to require low pH to initiate fusion (Helenius et al., 1982; Marsh et al., 1982). The SV40 polyoma virus uses caveolae and the more recently described non-clathrin non-caveolae route of entry. Amphotropic MLV (MLV-A) has recently been shown to prefer entry via caveolae endocytosis as well (Beer et al., 2005). Influenza virus can enter by both clathrin and the non-clathrin pathways but has a specific requirement for entry via a specific Rab 5 early endosome. Semliki forest and influenza viruses are pH-dependent and require entry via endocytosis but SV40 and MLV-A are pH-independent viruses so despite no dependence on delivery into an acidified vesicle to trigger fusion, these pH-independent viruses prefer endocytic entry. HIV is also a pH-independent virus that may, in some circumstances, use endocytosis for productive entry (Daecke et al., 2005; McClure et al., 1988).

The site of fusion for a pH-independent retrovirus can be the inside of an endosome or the cell surface given that there is a sufficient level of receptor/coreceptor at both sites. A virus can bind the cell surface and be 'taken up' by the cell coincident to a ligand triggered or constitutive endocytic process. It is also conceivable that a cell-free virus could be taken up with the fluid phase by macropinocytosis as has been postulated as a route of productive entry for HIV in MØs and microvascular endothelial cells (binding and fusion would then occur within the macropinocytic vesicle) (Liu et al., 2002; Marechal, 2001).

The various endocytic pathways used by HIV, and other viruses, to enter cells is discussed in more detail in chapter 4.

1.11.6 Early entry and reverse transcription events

The viral CA enters the cell and uncoats after fusion takes place. The contents of the capsid empty into the cell: **reverse transcription complex (RTC)** which includes **Integrase (IN)**, **reverse transcriptase (RT)**, **viral protein R (Vpr)**, and **matrix (MA)**, not to mention the two copies of the **RNA genome** (Bukrinsky et al., 1993b; Peterlin and Trono, 2003). Also released from the virion are **negative infectivity factor (Nef)**, **P6**, **Vpr**, and **viral infectivity factor (Vif)**, and the **capsid (CA)** protein of the now uncoated capsid (fig 1.4) [reviewed in (Cullen, 2001; Peterlin and Trono, 2003)].

The **RT** starts to reverse transcribe the **RNA genome** into a double-stranded complementary viral DNA for integration into the host genome, after cell entry but before nuclear translocation (Peterlin and Trono, 2003). For this to begin, the cortical actin cytoskeleton has been implicated as a scaffold for the formation of the RTC and initiation of reverse transcription (Bukrinskaya et al., 1998). Initial strong stop DNA has been reported to be reverse transcribed in the virion itself, before cell entry (Zhang et al., 1995), but the majority of viral DNA synthesis is thought to occur in the cytoplasm of the host cell (Bukrinsky et al., 1993b; Farnet and Haseltine, 1991; Karageorgos et al., 1993; Kim et al., 1989).

It has been shown that HIV uses the microtubule network for transport during entry (McDonald et al., 2002). More specifically, it has been suggested that a minus-end directed microtubule motor, dynein, might be used by HIV to shuttle the RTC towards the MTOC, which is adjacent to the nucleus (McDonald et al., 2002). This data on HIV is concurrent with what is known about the transport of adenoviruses and herpesviruses because Ad-2 and HSV-1 have been shown to use dynein for transport along microtubules to the nucleus (Dohner et al., 2002; Martin et al., 2002a; Sodeik, 2000; Sodeik et al., 1997; Suomalainen et al., 1999; Yedowitz et al., 2005).

Nef is packaged into the virion (Kotov et al., 1999; Welker et al., 1996), and this form of **Nef** has been shown to boost infectivity 10-fold over Δ Nef virions (Kotov et al.,

1999). It has been postulated that since an endocytosis directing VSV Env relieves HIV from its Nef dependence (Aiken, 1997), with an HIV Env, Nef may help direct the RTC to an endocytic location that is more conducive to reverse transcription and productive entry (Kotov et al., 1999).

1.11.7 Reverse transcription

Retroviral reverse transcription is highly error prone allowing a high rate of mutation and adaptation. The presence of two RNA molecules in the virion allows for crossover events during reverse transcription and can be especially advantageous if a virion is produced with two heterologous RNA strands from a superinfected cell. The RT enzyme is highly recombinogenic helping to facilitate this action. There is no proof reading mechanism in the RT enzyme or in the host RNA polymerase II that transcribes the integrated retroviral genome, resulting in an estimated 1 nucleotide mutation per 10^4 nucleotides per round of replication. The onward transmission of recombinant HIV viruses with mixed genomes from different clades is referred to as CRFs.

The Vif protein acts indirectly at this stage by preventing the packaging of apolipoprotein B mRNA-editing enzyme catalytic polypeptide (APOBEC) 3G, F and B into the viral particle at the stage of assembly (see also *virus budding* section) (Kao et al., 2003; Mariani et al., 2003; Marin et al., 2003; Mehle et al., 2004; Navarro and Landau, 2004; Sheehy et al., 2002; Sheehy et al., 2003). APOBECs 3G, F and B packaged with the virus particle cause cytosine to uracil deamination of the negative sense viral DNA during reverse transcription (Bishop et al., 2004a; Bishop et al., 2004b; Harris and Liddament, 2004; Mangeat et al., 2003; Newman et al., 2005; Sheehy et al., 2002; Stopak et al., 2003; Zheng et al., 2004). APOBECs packaged into the virion of Δvif viruses cause cytosine to uracil deamination resulting in nonsense mutation of the viral genome, and abortive replication (Harris and Liddament, 2004; Navarro and Landau, 2004).

RT reverse transcribes the packaged viral RNA into cDNA for integration into the host genome that is illustrated in fig 1.6.

After reverse transcription, IN remains bound to the viral cDNA and contains a nuclear localisation signal (NLS) that assists in nuclear translocation of the PIC (Bouyac-Bertoia et al., 2001).

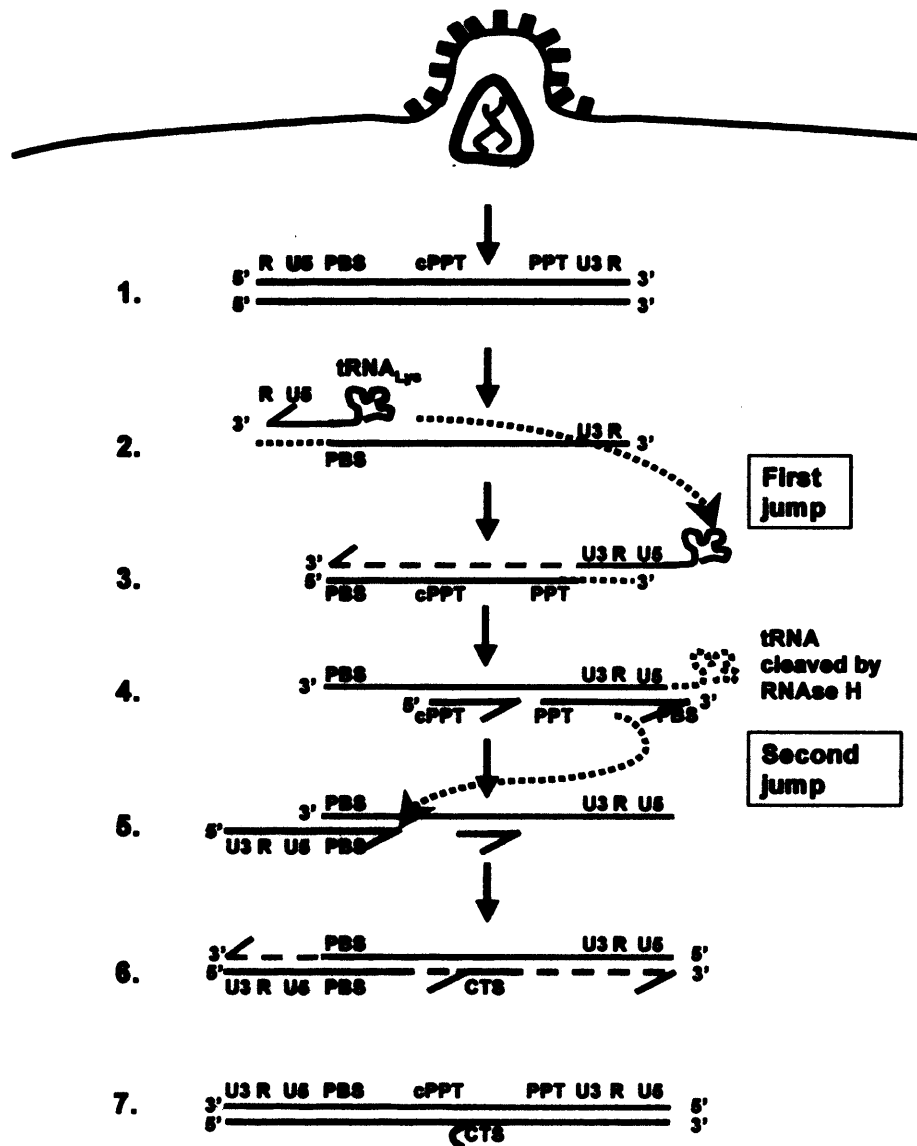


Figure 1.6. The process of reverse transcription.

Step 1: Plus sense, virion packaged RNA is shown in light blue, minus-sense DNA in orange and plus-sense DNA is shown in red. **Step 2:** Formation of negative-sense strong-stop DNA. RT is initiated from the paired 3' OH of a primer lysine-tRNA molecule (contained within the virion) complementary base paired to the viral RNA PBS. DNA is synthesised from the primer towards the 5' end of the RNA using the viral RNA as a template. The U5 and R regions are formed, RNase H activity degrades the RNA of the newly formed DNA/RNA hybrid, the tRNA molecule remains bound, and negative-sense

strong-stop DNA is formed. **Step 3: First jump.** RNase H activity in step 2 is required for the first jump to take place. The strong-stop DNA anneals to the R region at the 3' end of the RNA and formation of the minus strand DNA continues to the 5' end of the viral RNA molecule. 'Jumping' may go to the same viral RNA strand or may occur in *trans*, however there is evidence that jumping is completely random in this respect (Yu et al., 1998). Long minus strand DNA is formed and transcription normally stops in the vicinity of the PBS. RNase H activity degrades the RNA of the DNA/RNA hybrid when it is formed. **Step 4: Primer formation for plus strand DNA synthesis.** The central polypurine tract (cPPT) and the 3' PPT are resistant to RNase H activity and thus able to act as primers for the synthesis of the plus sense DNA strand, using the minus sense DNA strand as a template. DNA is copied into the primer binding sequence of the tRNA. When this happens RNase H degrades the tRNA. **Step 5:** The removal of the tRNA of the minus strand tRNA exposes the PBS on the plus strand DNA which allows the plus strand molecule to anneal to the PBS at the 3' end of the minus strand DNA in the second jump. **Step 6:** The second jump annealing forms a circular structure (not shown) and both minus and plus sense strands are completed. **Step 7:** Reverse transcription is completed with linearising of the circular structure and formation of a 99 nt central DNA flap. In Lentiviruses, RT is terminated at the central termination sequence (CTS) beyond the cPPT, which results in the formation of a 99 nt DNA flap (Charneau et al., 1994)

1.11.8 Retroviral restriction and virus entry

Ref1, Lvl and Fv1 are retroviral restriction factors in human, simian and mouse cells, respectively [reviewed in (Bieniasz, 2003; Bieniasz, 2004)]. These factors terminate retroviral replication at the entry stage. However, where Fv1 allows reverse transcription to be completed, Ref1 and Lvl block replication before reverse transcription (Bieniasz, 2003). Human and rhesus macaque TRIM 5 α have been shown to be responsible for the majority of Ref1 and Lvl restriction, respectively, and are thought to reside in cytoplasmic bodies within the cell (Stremlau et al., 2004). The anti-MLV restriction factor Fv1 has been shown to localise to a microtubule localised compartment, adjacent to the trans-Golgi network (Yap and Stoye, 2003), so the different cellular localisations of these factors may influence whether or not reverse transcription is blocked during restriction.

The action of retroviral restriction factors in intrinsic immunity is discussed in more detail in chapters 5 and 6.

1.12 Into the nucleus...

1.12.1 Nuclear import of the pre-integration complex

At the end of reverse transcription there is an apparent change in the protein composition of the RTC. The sub-viral unit, at the completion of reverse transcription, is referred to as the pre-integration complex (PIC), and this may shed the RT and MA that were associated with the RTC and remain associated only to IN (Farnet and Haseltine, 1991; Karageorgos et al., 1993). So it has been argued that the PIC may only require the NLS of IN for translocation across the nuclear envelope in non-dividing cells (Farnet and Haseltine, 1991; Karageorgos et al., 1993). The size and the proteins that associate with the HIV PIC are, however, controversial as will be discussed later.

As entry progresses, smaller RTC species appear and the smallest species localise to the nucleus at late post-entry time points, indicated by decreasing sedimentation ('S') values (Fassati and Goff, 2001; Karageorgos et al., 1993), this alteration in size may assist the PIC to traverse the small 25 nm pore size of the NPC. Reports of the PIC nucleoprotein complex size vary from 56 nm (Sherman and Greene, 2002), to sizes of 400 to 700 nm (McDonald et al., 2002) and 200nm (Nermut and Fassati, 2003). Nevertheless, the PIC is complex and probably requires the action of several NLSs to effect nuclear entry (Bukrinsky and Haffar, 1999) including those of cellular proteins that have been recruited to the viral PIC (Maertens et al., 2004). In addition, nuclear import/export is a redundant system and PIC nuclear translocation may involve numerous nuclear import pathways (Jenkins et al., 1998; Kuersten et al., 2001).

Vpr is packaged into virus particles through its interaction with the C-terminal p6 domain of the p55 Gag polyprotein (fig 1.5 and 1.11), at roughly 14 molecules per virion (Sherman and Greene, 2002). Vpr has been shown to induce "disruptions" in the nuclear envelope, which may facilitate trafficking of the 56-700 nm PIC across the nuclear membrane where nuclear pores are normally only 25nm across (de Noronha et al., 2001). Vpr may also utilise its two novel NLSs for PIC nuclear import, one of which results in the direct association of Vpr with the nuclear pore complex (NPC) (Jenkins et al., 1998).

Beside its role for providing structure to the virus particle, MA, like Vpr, displays nucleocytoplasmic shuttling and is thought to contribute to the nuclear localisation of the PIC (Jenkins et al., 1998). MA is a part of the PIC and contains a classic NLS that is thought to contribute to nuclear localisation (Jenkins et al., 1998; Peterlin and Trono, 2003). It has been shown that lentiviral nuclear localisation in non-dividing cells requires the NLS of MA (Bukrinsky et al., 1993a; Bukrinsky et al., 1993b; Dubrovsky et al., 1995), however there are other reports that claim MA is not required for PIC nuclear translocation (Farnet and Haseltine, 1991; Fouchier et al., 1997).

A 99 nt DNA flap, formed by strand displacement at the cPPT just before RT termination at the CTS (fig 1.6; step 7) (Zennou et al., 2000), has been shown to enhance nuclear localisation of lentiviral vectors (Dardalhon et al., 2001; Sirven et al., 2000). Later studies have shown that the 99 nt flap may be repositioned at a wide range of locations within the viral cDNA for enhancement of nuclear localisation (De Rijck et al., 2005).

Viral protein x (Vpx) is an accessory gene found in viruses of the HIV-2/SIV_{SMM}/SIV_{MAC} group of primate lentiviruses, but not HIV-1 (fig 1.3). Vpx is proposed to have arisen as the result of a gene duplication of Vpr since the two genes share sequence homology (Tristem et al., 1990). The gene redundancy of Vpx and Vpr does not equate to a functional redundancy since the roles of Vpr in HIV-1 have been split-up in HIV-2 Vpx and Vpr (Fletcher et al., 1996): the PIC nuclear localisation and G2-M cell cycle arrest activities of HIV-1 Vpr are delegated to HIV-2 Vpx and Vpr, respectively (Fletcher et al., 1996), and the Vpx NLS has been shown to be necessary and sufficient for nuclear localisation of the HIV-2 PIC into the nucleus in non-dividing cells (Belshan and Ratner, 2003).

1.12.2 Cellular genes involved in HIV nuclear import

The importins mediate the majority of traffic into and out of the nucleus by interacting with the RanGTP/GDP system which is governed by the RanGTP/GDP concentration gradient between the nucleus and cytoplasm; low levels of RanGTP in the cytoplasm allow the binding of importins to their cargoes, whereas high RanGTP levels in the

nucleus induce their dissociation [reviewed in (Gorlich and Kutay, 1999)]. It is therefore conceivable that one of these proteins may mediate the nuclear translocation of HIV-1 PICs. Indeed, **Importin 7**, a member of the importin β superfamily, has been implicated in the nuclear import of HIV-1 PICs into the nucleus (Fassati et al., 2003).

Lens epidermal growth factor (LEDGF) is a cellular protein that has been shown to associate with and promote the nuclear localisation of the PIC (Maertens et al., 2004), and chromosome tethering of IN (Emiliani et al., 2005).

1.12.3 Integration

Integration is the process whereby the viral cDNA genome is inserted into the host DNA genome. IN remains associated with the PIC after translocation into the nucleus and performs the integration reaction.

Following reverse transcription, the viral cDNA is “primed” for integration in the cytoplasm by IN mediated 3’ trimming of the DNA ends, termed ‘3’ processing’ (fig 1.7) [reviewed in (Pommier et al., 2005)]. Trimming is a 3’ endonucleolytic cleavage of 2 bases exactly 3’ of a CA motif that creates 3’ CA ends on the viral DNA (Pommier et al., 2005). The 3’ CA-OH ends are 3’ reactive intermediates required for strand transfer to occur (Pommier et al., 2005).

Following entry into the nucleus, IN catalyses the insertion of the viral DNA into a host chromosome. IN remains bound to the viral cDNA as a multimeric complex bridging the viral DNA ends. The 3’-OH ends of the PIC are joined to the 5’ ends of a host chromosome, catalysed by IN (fig 1.7). This joining event leads to a canonical five base stagger. Completion of integration requires trimming of two nucleotides from the 5’ proviral ends and gap filling from the 3’-OH chromosomal DNA ends (fig 1.7). It is likely that cellular enzymes are involved in 5’ processing however their identity remains uncertain (Daniel et al., 2004; Yoder and Bushman, 2000).

Cellular proteins have been shown to have IN binding and catalysis of integration activities (Pommier et al., 2005). **Ini1 (Integrase interactor protein 1)** was the first discovered cellular IN binding protein that promotes integration (Turelli et al., 2001;

Turlure et al., 2004). Since then, **heat shock protein 60 (Hsp60)** has been shown to bind IN and stimulate *in vitro* processing and end-joining (Parissi et al., 2001), **LEDGF** is an IN to chromatin tethering factor that is not absolutely necessary for integration but may play a chromosomal DNA targeting role (Cherepanov et al., 2003; Llano et al., 2004), **embryonic ectoderm development protein (EED)** has been suggested to help traffic or “convoy” the PIC to the nucleus, during entry (Violot et al., 2003), and **radiation sensitivity protein 18 (Rad 18)** may be involved in the post-integration “translesion repair” that must take place to seal the gap between the viral 5’ and chromosomal 3’ DNA ends (fig 1.7c) [(Mulder et al., 2002) reviewed in (Turlure et al., 2004)].

Cellular proteins **barrier to auto-integration factor (BAF)** (Lee and Craigie, 1998; Lin and Engelman, 2003), and **high mobility group protein A1 (HMGA1)** bind DNA directly and regulate integration of HIV and MLV [(Li et al., 1998; Li et al., 2000) reviewed in Pommier et al, 2005]. HMGA1 stimulates IN activity either by condensation of reactant DNA or the binding of HMGA1 monomers to multiple DNA sites may fold reactant DNA into conformations that favour IN catalysis and assembly (Farnet and Bushman, 1997; Li et al., 1998; Li et al., 2000). BAF stimulates intermolecular integration by binding the double stranded DNA of the PIC (rather than intramolecular integration resulting in 2 LTR circles) (Harris and Engelman, 2000; Lee and Craigie, 1998).

PML (TRIM 19) has been shown to associate with HIV-1 RTCs in the target cell (in the cytoplasm) and interfere with integration (Turelli et al., 2001).

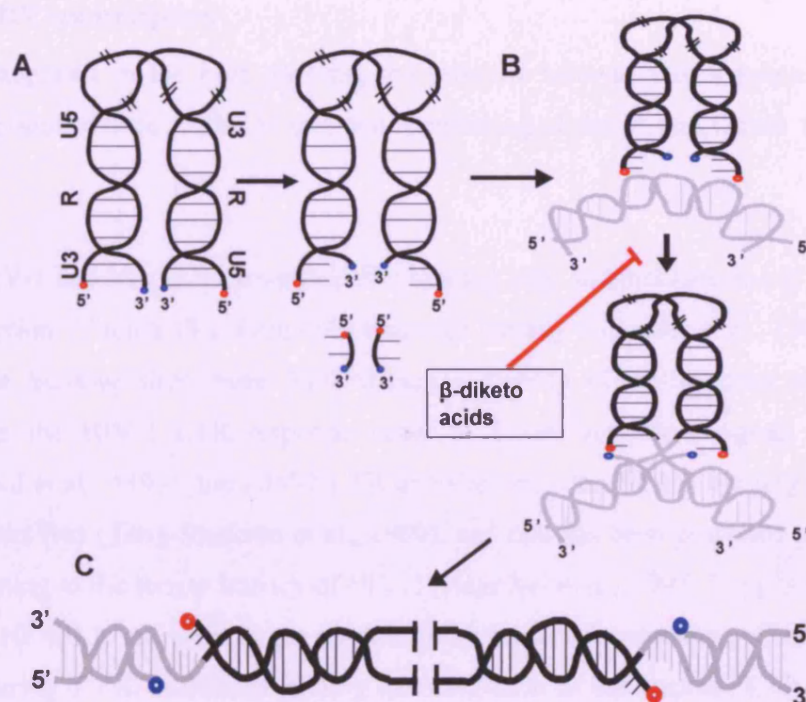


Figure 1.7. HIV integration.

(A) 3' processing: viral cDNA genome is shown in black and the host genomic DNA is grey, 3' ends are denoted by blue circles and the 5' ends are denoted by red circles. IN catalyses an endonucleolytic cleavage of two bases from the 3' CA ends. HIV-2 is unique among the lentiviruses, and indeed the *Retroviridae*, in that an asymmetric 3 bases are removed from the U5 LTR and 2 bases from the U3 LTR (Whitcomb and Hughes, 1991). (B) Strand transfer is catalysed by IN mediated ligation of the viral 3'-OH ends (created in the 3' processing reaction) to 5' phosphate groups of the host DNA. (C) This joining event leads to a canonical five base stagger. Completion of integration by gap filling from the 3'-OH chromosomal DNA ends (Pommier et al., 2005).

1.12.4 Integration site selection

In vitro integration systems have shown that IN prefers to integrate the viral genome into severely kinked DNA regions within the nucleosome (Pruss et al., 1994a). The curvature induced by the nucleosome may widen the DNA major groove and thus promote the interaction of host DNA with IN (Pruss et al., 1994b). Human genome-wide screens have shown that HIV-1 integration favours active genes (Schroder et al., 2002; Wu et al., 2003) whereas MLV favours integration in transcription start regions (Wu et al., 2003).

1.12.5 HIV transcription

Once integrated in the host genome, the provirus behaves like a human gene with transcription initiated at the 5' end and terminating at the 3' end (Jones and Peterlin, 1994).

Both HIV-1 and HIV-2 have very similar binding sites and mechanisms of LTR driven transcription. Their LTRs share 40% sequence identity (Guyader et al., 1987) and their promoter binding sites share 50% sequence identity (Tong-Starksen et al., 1990). However the HIV-1 LTR responds better to T cell activation signals than HIV-2 (Hannibal et al., 1993); the HIV-2 LTR contains only one NF- κ B binding site whereas HIV-1 has two (Tong-Starksen et al., 1990), and this has been proposed to be a factor contributing to the longer latency of HIV-2 (Hannibal et al., 1993; Tong-Starksen et al., 1990). NF- κ B is activated in the cytoplasm by proteolytic cleavage of inhibitor of κ B (I κ B) during T cell activation leading to localisation to the nucleus, LTR binding and up-regulation.

The majority of the HIV promoter is contained within the U3 region, with reported SP1 transcription factor binding sites in the HIV-1 leader region (Logan et al., 2004). The LTR contains enhancer and promoter regions with binding sites for several transcription factors and a polyadenylation signal (fig 1.8) (Marciniak et al., 1990; Southgate and Green, 1991). Moving upstream from the transcription start site, the initiator (Inr), the TATA box, and 3 SP-1 sites are found (Berkhout and Jeang, 1992). These sites position RNA polymerase II at the correct site for initiating transcription (Berkhout and Jeang, 1992). The NF-AT and NF- κ B transcription factor binding sites allow maximum levels of viral transcription in activated T cells and M ϕ s (fig 1.8) (Griffin et al., 1989; Griffin et al., 1991; Jones and Peterlin, 1994).

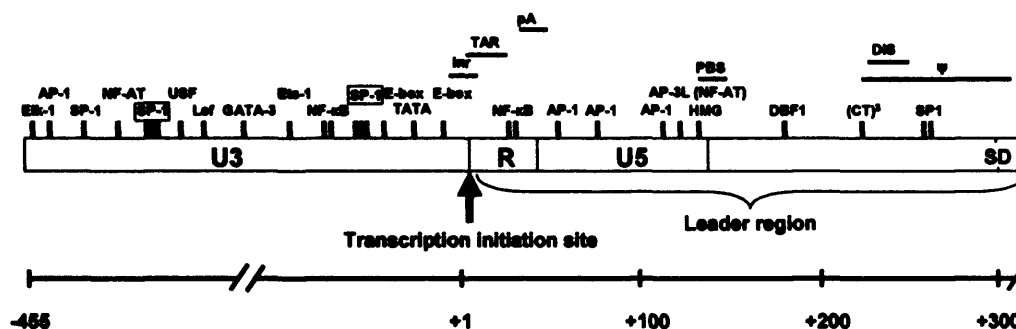


Figure 1.8. Promoter and primer binding sites and key RNA structural features of the HIV-1 LTR.

Key promoter binding sites are shown in addition to the polyadenylation site (pA), the position of the RNA TAR loop (TAR), transcription initiator (Inr), primer binding site (PBS), dimer initiation site (DIS), and the ψ packaging signal. SD = *gag* splice donor site. The LTR is not drawn to scale (Logan et al., 2004).

Early events of HIV transcription involve **transactivator of transcription (Tat)** and **regulator of expression of viral proteins (Rev)**. HIV remains in a transcriptionally latent state after integration with only the appearance of short TAR transcripts in the nucleus (Adams et al., 1994; Feinberg et al., 1991). Cellular activation signals improve the rate of transcription and full-length, fully spliced 2 kb mRNA transcripts appear (fig 1.10) (Jones and Peterlin, 1994). These 2 kb mRNA species encode for Tat, Rev and Nef (fig 1.10) (Kim et al., 1989). Tat and Rev both contain NLSs so after translation they are transported back into the nucleus and dramatically improve the processivity and fidelity of HIV transcription (Malim et al., 1989a; Perkins et al., 1989; Ruben et al., 1989). Tat then interacts with TAR and greatly improves the quantity of polyadenylated genome length transcripts in the nucleus (fig 1.9)(Adams et al., 1994; Feinberg et al., 1991).

1.12.6 Mechanism of Tat transactivation

The TAR element, which forms an RNA stem loop, is the target RNA sequence for Tat transactivation (Feng and Holland, 1988; Muesing et al., 1987; Peterlin et al., 1986). Tat in conjunction with positive transcription elongation factor B (p-TEFb), binds TAR with high affinity that allows elongation of viral RNA transcripts through activation of

RNA polymerase II (fig 1.9a) (Kao et al., 1987; Wei et al., 1998). The p-TEFb complex consists of cyclin T1 and CDK9 (fig 1.9b) (Wei et al., 1998), and once recruited to the nascent RNA complex, CDK9 phosphorylates the C-terminal domain of RNA polymerase II and negative transcription elongation factor (N-TEF) (Yamaguchi et al., 1999), leading to high fidelity and processive transcription (fig 1.9c) (Peterlin and Trono, 2003; Price, 2000).

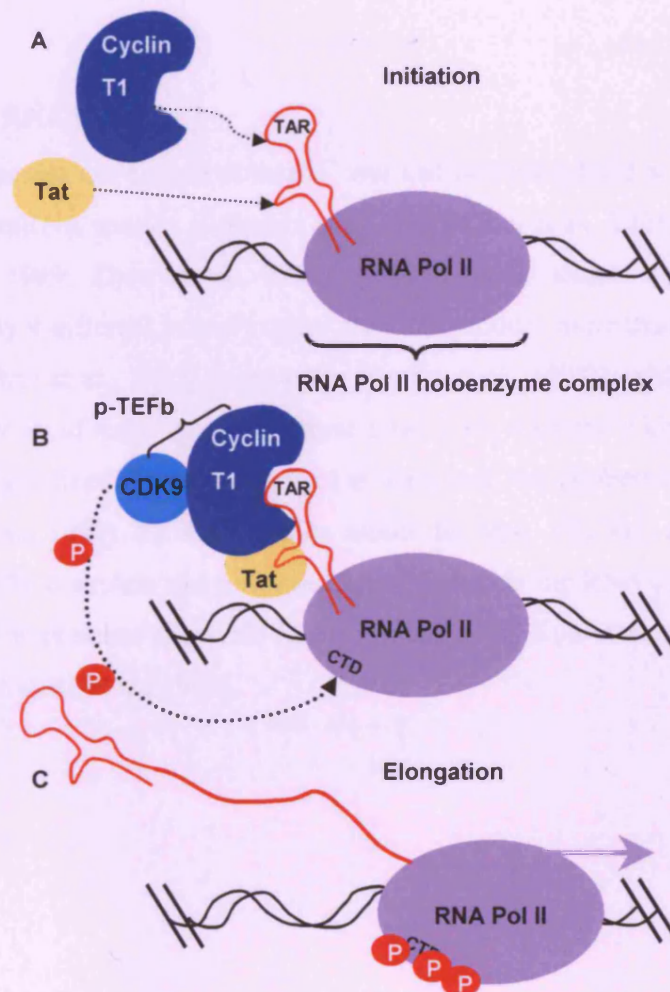


Figure 1.9. HIV transcription: the initiation complex.

(A) The RNA polymerase II holoenzyme complex (RNA Pol II) binds to upstream basal transcription promoters in U3 of the LTR (fig 1.8) and, without Tat, transcription will begin but elongation is inefficient. The nascent TAR RNA folds into the TAR stem loop structure and recruits cyclin T1 and Tat. (B) Tat binds the 5' bulge and Cyclin T1 binds the central loop of TAR. CDK9 of the p-TEFb complex phosphorylates the C-terminal domain (CTD) of RNA Pol II (C), which results in the displacement of TAR from the polymerase, and efficient transcription/elongation of the viral genome by RNA Pol II (Jones and Peterlin, 1994; Karn, 1999).

Tat functions poorly in mouse cells, however mouse human chromosome 12 somatic hybrids have been shown to recover Tat transactivation (Hart et al., 1989; Hart et al., 1995; Newstein et al., 1990). An 83 kDa protein expressed from human chromosome 12 was identified as human Cyclin T1, the cofactor required for efficient Tat transactivation (Hart et al., 1995). A Cyclin T1 homologue is encoded by mice but it does not support Tat and TAR interactions like its human homologue (Bieniasz et al., 1998; Wei et al., 1998).

1.12.7 Viral RNA species

HIV RNA species are capped at their 5' end and polyadenylated at their 3' end much like cellular mRNA species (Bohnlein et al., 1989; Chiu et al., 2001; Chiu et al., 2002; Das et al., 1999; Zhou et al., 2003). The genome length viral RNA contains approximately 9 different primary splice sites that produce more than 30 different RNA species [(Bohne et al., 2005) reviewed in (Coffin et al., 1997)], which can be detected in the cell. Most of these species are three sizes; 2 kb, 4 kb and 9 kb species (fig 1.10): the 2 kb fully spliced class encode for Tat, Rev, and Nef (Robert-Guroff et al., 1990; Schwartz et al., 1990), the 4 kb species encode for Vpu, Vif, Vpr, and Env (Arrigo et al., 1990). The complete and unspliced species provide the RNA coding sequence for Gag and Pol expression (fig 1.10) (Arrigo et al., 1990; Kim et al., 1989; Neumann et al., 1994; Schwartz et al., 1990).

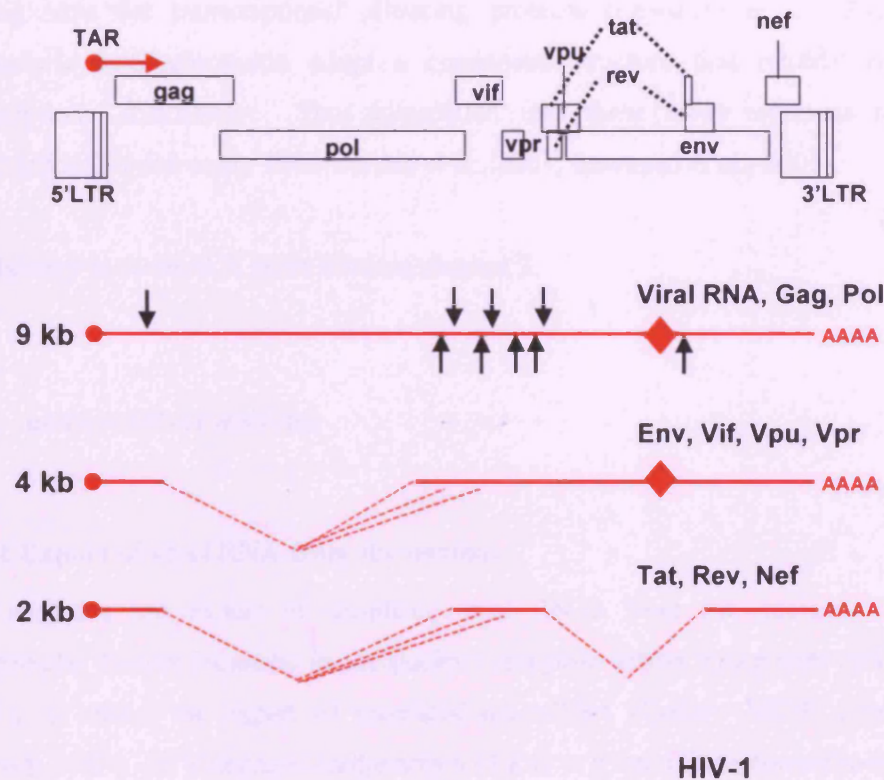


Figure 1.10. HIV-1 messenger RNA expression.

The three mRNA species present during active infection are shown in red. TAR is present in all of the species (solid red dot) and the Rev response element (RRE) is shown as a red diamond in the 4 and 9 kb RNAs. The splice donor sites are the arrows pointing down and the splice acceptor sites are the arrows pointing up. The 2 kb 'early' transcripts produced first do not require the action of Rev and the RRE because they are fully spliced. The full-length and partially spliced late transcripts are produced by the assistance of Tat and Rev. Positions are approximate. Derived from (Arrigo et al., 1990; Dalglish and Weiss, 1999).

Sites of HIV gene transcription repression have been mapped and one study has revealed that repression of HIV transcription is associated with gene deserts, centromeric heterochromatin and very highly expressed cellular genes (Lewinski et al., 2005). Even though HIV integration has been shown to prefer transcriptional units integration into very highly expressed genes can silence provirus transcription [(Lewinski et al., 2005; Schroder et al., 2002; Wu et al., 2003) reviewed in (Bushman et al., 2005)]. Read-through transcription of cellular genes into provirus can dislodge HIV transcription factors, or "train wrecking", the colliding of converging RNA polymerase complexes (Lewinski et al., 2005). Gene deserts however may be enriched with

binding sites for transcriptional silencing proteins (Lewinski et al., 2005), and centromeric heterochromatin adopt a condensed structure that inhibits access to transcriptional machinery. Thus integration into these areas represses retroviral transcription (Jordan et al., 2003; Jordan et al., 2001; Lewinski et al., 2005).

HIV latency is covered in more detail in chapter 3.

1.13 ...and out (of the nucleus)

1.13.1 Export of viral RNA from the nucleus

Rev mediates the export of unspliced viral RNAs from the nucleus. Cellular commitment factors localised in the nucleus recognise intron splice sites and monitor mRNA to inhibit the export of unspliced pre-mRNA (Cullen, 2003b; Legrain and Rosbash, 1989). HIV depends on the action of Rev to bypass these commitment factors and export the unspliced 4 and 9 kb late viral RNA species from the nucleus (Malim et al., 1989a; Malim et al., 1989b). Rev usurps control over nuclear export inhibition of unspliced RNA species by binding the RRE that lies within the *env* gene, thus promoting the export of unspliced viral RNA (Fischer et al., 1995; Malim et al., 1989b). The RRE is a 240 bp long stretch of complex secondary RNA structure that interacts with the Rev arginine-rich RNA binding motif (ARM) (Malim et al., 1990; Zapp and Green, 1989). Export of viral mRNA is driven by interaction with the cellular CRM1 protein known to export host U snRNA and rRNAs (Bogerd et al., 1998; Fornerod et al., 1997; Neville et al., 1997; Stade et al., 1997), this is intriguing since the CRM1 pathway isn't thought to be an export pathway for cellular mRNAs (Cullen, 2003b).

Step 1: in order for CRM 1 to bind the viral export cargo (Rev: RRE-RNA) it must first bind the Ran-GTP nuclear export factor (Askjaer et al., 1998; Fornerod et al., 1997). Multiple Rev molecules assemble onto the RRE-RNA using the ARM sequence and recruit several CRM 1 Ran-GTP complexes to the RRE (Askjaer et al., 1998; Fischer et al., 1995; Fornerod et al., 1997; Henderson and Percipalle, 1997; Malim et al., 1989a; Malim et al., 1989b; Malim et al., 1990; Olsen et al., 1990).

Step 2: CRM 1 targets the viral ribonucleoprotein complex to the nuclear pore (Cullen, 2003a; Fornerod et al., 1997; Neville et al., 1997), where the CRM 1-Ran-GTP-Rev: RRE-RNA complex traverses the nuclear envelope to the cytoplasm.

Step3: in the cytoplasm, Ran BP1 and Ran GAP induce the hydrolysis of Ran-GTP to GDP, triggering the release of CRM 1 from the Rev NES, exposing the Rev NLS and as a result, Rev in addition to CRM 1 are transported back into the nucleus (Askjaer et al., 1998; Cullen, 2003a; Fischer et al., 1999; Henderson and Percipalle, 1997). The incompletely spliced viral RNA, now in the cytoplasm, is available for translation by ribosomes or packaging into virions.

1.13.2 Particle formation and budding

Once the HIV viral proteins have been translated they must then be assembled in the cytoplasm, and trafficked to the site of budding. This requires targeting of viral proteins so that all constituents, required to make an infectious particle, are assembled properly and in the same place. From the point of view of a handful of different viral proteins, the inside of the cell is a very large and viscous place (300mg protein/ml) (Sodeik, 2000). Molecules are therefore incapable of 1, diffusing freely through the cytoplasm to reach the point of assembly and 2, randomly assembling into a highly organised viral particle. This section will discuss the recruitment of cellular protein expression and budding machinery by HIV to transport viral particle constituents and assemble them into full-infectious viral particles. The mechanism of viral protein assembly will also be discussed.

Full-length/uncleaved **Gag-Pol** and **p55-Gag** (fig 1.11) precursors play major roles during assembly and form the bulk of virion structure. It has been estimated that the Gag shell of the immature retrovirus particle is built up of 1500-1800 Gag monomers (Adamson and Jones, 2004). However the retroviruses only require about 1 Pol for every 10-20 Gag proteins so Gag-Pol production must be less frequent or efficient than Gag production [reviewed in (Coffin et al., 1997)]. HIV therefore prevents Pol read-through by a retroviral mechanism called “frameshift suppression”, where the ribosome must slip back –1 nucleotides in order to complete the Gag-Pol precursor protein (Jacks et al., 1988; Wilson et al., 1988). The *gag* and *pol* genes of HIV are out of translational

phase and -1 frameshifting puts them back into the same reading frame, essentially increasing the size of the *gag* ORF to include *pol*. The 0 to -1 frameshift occurs at a specific codon of the RNA, 5' of the *gag-pol* overlap region (fig 1.3) (Jacks et al., 1988; Wilson et al., 1988). MLV uses a read-through suppression mechanism called "termination suppression" whereby the ribosome reads through the *gag* termination codon as though it were a sense codon. Therefore MLV *gag* and *pol* are encoded in the same reading frame (fig 1.3). The efficient packaging of Pol (Pr, RT and IN) by orthoretroviruses depends upon fusion with Gag since the targeting sequence for budding is within Gag and not Pol. In fact, expression of Gag and Gag-Pol alone will drive the formation of retrovirus-like particles that are indistinguishable from full infectious particles (Coffin et al., 1997).

1.13.3 RNA encapsidation

Encapsidation of genomic length HIV-1 and HIV-2 RNA depends upon the binding of the RNA packaging signal Ψ to dual zinc fingers of NC within p55-Gag [(Aldovini and Young, 1990; Dannull et al., 1994; Dorman and Lever, 2000; South and Summers, 1993; Tsukahara et al., 1996) reviewed in (Coffin et al., 1997)]. Only genome length viral RNAs of HIV-1 contain the Ψ signal because Ψ exists in the leader region of the genome, downstream of the *gag* SD site. However, all RNA transcripts of HIV-2 contain the Ψ signal since it lies upstream of the *gag* SD site (Griffin et al., 2001; Kaye and Lever, 1998; Kaye and Lever, 1999; McCann and Lever, 1997). In this instance, HIV-2 uses a novel mechanism to select for genome length viral RNAs: cotranslational packaging of the RNA by the nascent NC and competition for limiting concentrations of Gag polyprotein (Griffin et al., 2001), so translation of *gag* and packaging of RNA occur cotranslationally. This is in contrast to simple retroviruses, such as MLV, where the RNA that is translated is compartmentally separate from the RNA that is encapsidated (Levin and Rosenak, 1976).

During budding, Gag egress requires the binding of RNA via Ψ for transport along actin microfilaments (Mouland et al., 2001; Poole et al., 2005; Rey et al., 1996), suggesting an intracellular targeting role for the RNA, whereas microtubules have been implicated in trafficking of RTCs during viral entry (McDonald et al., 2002).

The MA region of p55-Gag forms the final contacts with the inside of the virion. It does this via the M domain that is myristoylated in order to target Gag to the PM and/or membrane surfaces within the cell (figs 1.4 and 1.11) (Adamson and Jones, 2004).

1.13.4 Virus budding and the endosomal sorting complex required for transport 1 machinery

The type-C retroviruses, which include the alpha and gamma retroviruses and Lentiviruses, assemble their cores at the PM (fig 1.4, step 7) [reviewed in (Demirov and Freed, 2004)]. The type-B, D and spuma retroviruses assemble their cores in the cytoplasm. The fully assembled cores, known as intracytoplasmic A-type cores, are then trafficked to the PM for budding and particle release (Coffin et al., 1997).

HIV and type-C retroviruses like MLV depend upon interactions with cellular **endosomal sorting complex required for transport (ESCRT) machinery** to effect budding from cell membranes. The p6 and p12 proteins of Gag are required for particle budding in HIV and MLV, respectively (Gottlinger et al., 1991; Huang et al., 1995). The PTAP and PPPY motifs in p6 of HIV and p12 of MLV, respectively, have been shown to be absolute requirements for particle release and have been aptly named the 'late domains' (fig 1.11) (Gottlinger et al., 1991; Huang et al., 1995; Wills and Craven, 1991; Yuan et al., 2000; Yuan et al., 1999). The requirement of the late domain for particle release is illustrated by the incomplete budding and 'tethering' of virus particles to the surface of the producer cell ("stalk mutants") when the PTAP motif of HIV-1 is mutated (Gottlinger et al., 1991). The host cell protein TSG101 interacts with the PTAP motif in the L domain of p6 in HIV-1 Gag (fig 1.11) (Garrus et al., 2001; VerPlank et al., 2001), which couples virion formation to the cellular **ESCRT** trafficking machinery (Katzmann et al., 2001; Pornillos et al., 2002; Vogt, 2000). Depletion of TSG 101 does not inhibit budding by MLV but experiments inhibiting **VPS 4** activity block both HIV and MLV budding (Garrus et al., 2001).

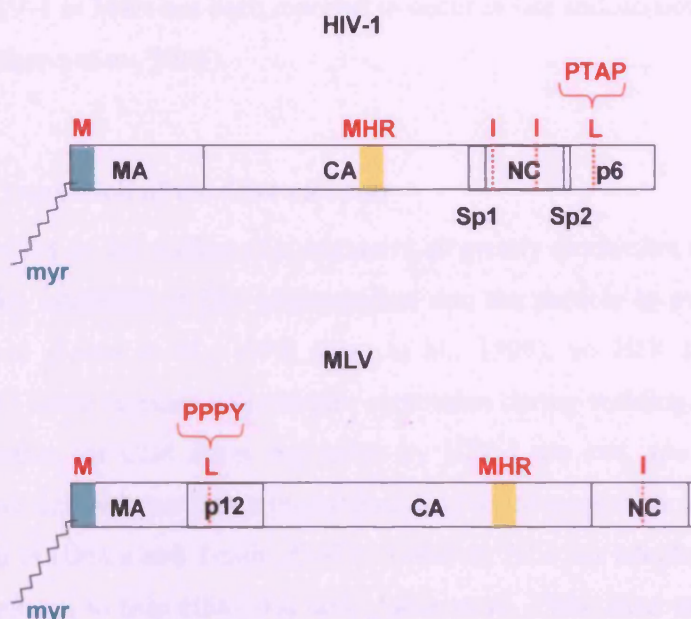


Figure 1.11. HIV-1 p55 and MLV p65 Gag polyprotein schematic.

Uncleaved Gag polyprotein showing regions important for virus budding. M = membrane association domain of Gag that is myristoylated (myr) at the N-terminus. MHR = major homology region, present in the capsid of all retroviruses is important for Gag-Gag interactions during budding, however it is absent in the spumaviruses (Maurer et al., 1988; Renne et al., 1992). I = interaction domains important for Gag-Gag and Gag- ψ RNA interactions during particle assembly. Basic residues are important for Gag-Gag interaction and the Zn^{+2} fingers formed in these domains are important for interaction with RNA (Lee and Linial, 2004). L = late domain shown to bind TSG 101 (HIV-1) for particle budding via the ESCRT 1 machinery. Sp1 and Sp2 = spacer peptide 1 and 2.

The PTAP motif of HIV-1 p6 Gag binds the N-terminal region of TSG 101. The C-terminal portion of TSG 101 binds VPS 28 such that prevention of VPS 28 to TSG 101 interaction abolishes the ability of TSG 101 to mediate particle budding (Martin-Serrano et al., 2003). An ATPase called VPS 4 has also been implicated as an additional factor required for budding because ATPase defective VPS 4 mutants inhibit HIV-1 and MLV budding (Garrus et al., 2001), which is consistent with an earlier finding that depletion of ATP potently inhibits budding (Tritel and Resh, 2001). TSG 101 and VPS 28 are normally associated within the cytoplasm for regulation of traffic to and from the multi vesicular body (MVB) and late endosomes, but their interaction with HIV-1 Gag results in the budding of particles from the PM (Martin-Serrano et al., 2003). Moreover, consistent with the normal localisation of TSG 101 and VPS 28,

budding of HIV-1 in MØs has been reported to occur in late endosomes and not the PM (Pelchen-Matthews et al., 2003).

1.13.5 Down regulation of the CD4 receptor

An excess of CD4 on the surface of a producer cell greatly diminishes the infectivity of virus, either by inhibition of Env incorporation into the particle or even inhibition of particle release (Lama et al., 1999; Ross et al., 1999), so HIV has incorporated mechanisms to down regulate cell receptor expression during budding. The three viral genes responsible for CD4 down regulation by HIV-1 are *env*, *vpu* and *nef*. Most viruses achieve cell receptor down modulation by Env sequestration of receptor in the ER [reviewed in (Doms and Trono, 2000)]. However HIV has adapted two additional genes, *vpu* and *nef*, to help effect this task (Aiken et al., 1994; Bour et al., 1995; Chen et al., 1996a; Margottin et al., 1998; Rhee and Marsh, 1994).

Nef carries out its functions by establishing connections between proteins. It has no enzymatic activity and is essentially a conglomerate of protein-protein interaction domains (Doms and Trono, 2000; Peterlin and Trono, 2003). Nef down regulates cell surface CD4. During CD4 down modulation, Nef acts as a connector to the cytoplasmic tail of CD4 and clathrin adaptor proteins, thereby directing CD4 into a clathrin-mediated degradative pathway (Aiken et al., 1994; Bresnahan et al., 1998; Foti et al., 1997; Margottin et al., 1998; Schwartz et al., 1995). Considerably less is known about the function of HIV-2 Nef, but this protein has also been shown to down modulate the expression of CD4 and MHC-I (Munch et al., 2005).

Vpu acts in a very similar way to Nef in that it too is an adaptor for directing CD4 into a degradative pathway. Vpu bridges the cytoplasmic tail of Env trapped CD4 receptors, in the ER, to proteolysis machinery. Vpu bound to CD4 binds a protein called β TrCP, which in turn links this protein complex to the proteasome (Bour et al., 1995; Margottin et al., 1998; Schubert et al., 1998), and Env is then free to make its way to the cell surface (Bour and Strebel, 2003; Willey et al., 1992).

Vpu is also thought to improve particle release via a poorly defined, CD4 and Env independent mechanism (Paul et al., 1998; Schubert et al., 1996; Schubert and Strebel,

1994). It has been suggested that Vpu enhances particle production by counteracting a cell-type dependent virus assembly restriction (Varthakavi et al., 2003).

HIV-2 does not encode for Vpu (fig 1.3), however enhancement of HIV-2 virus particle release, has been attributed to the cytoplasmic tail of HIV-2 Env (Bour et al., 1996; Bour and Strebel, 1996; Ritter et al., 1996). A membrane proximal GYXX motif in the cytoplasmic domain has been reported as necessary for this activity (Abada et al., 2005).

1.13.6 Immune evasion

Nef decreases the expression of MHC-I molecules from the cell surface, helping virus infected cells to avoid recognition and killing by CTLs (Peterlin and Trono, 2003). Since Nef is an early expressed protein (fig 1.10) it is a well-chosen candidate to prevent immune recognition by MHC-I. The rest of the HIV replication cycle may proceed uninterrupted because the bulk of virus protein production can then proceed without the pressure of virus protein being presented by MHC-I to cytotoxic T cells at the cell surface. Nef appears however, to be selective in its down regulation of only HLA-A and B, but not HLA-C or HLA-E, perhaps to avoid detection from a lack of MHC expression and subsequent killing by NK cells (Cohen et al., 1999; Kasper and Collins, 2003; Williams et al., 2005; Williams et al., 2002).

1.13.7 Apolipoprotein B mRNA-editing enzyme catalytic polypeptide restriction of HIV and MLV

Vif acts at the point of assembly by promoting the degradation of hAPOBEC 3G proteins that would otherwise be packaged into the virion (Sheehy et al., 2002; Sheehy et al., 2003). Cytidine deamination of the RT minus strand DNA by hAPOBEC 3G during RT then occurs upon entry of the RTC, inhibiting virus replication (see also *reverse transcription* section) (Bishop et al., 2004a; Bishop et al., 2004b; Mangeat et al., 2003; Newman et al., 2005). In this regard Vif acts directly at the point of assembly but indirectly at entry and RT.

Vif and **hAPOBEC 3G** co-immunoprecipitate suggesting that they bind one another (Mariani et al., 2003; Marin et al., 2003; Sheehy et al., 2003; Stopak et al., 2003). One report has shown that Vif may inhibit hAPOBEC 3G translation from mRNA in infected cells (Stopak et al., 2003). Proteasome inhibitors can partially reverse the effect of Vif mediated decrease on hAPOBEC 3G steady state levels and Vif-APOBEC complexes can then be seen packaged into virions (Kao et al., 2003; Marin et al., 2003; Mehle et al., 2004; Sheehy et al., 2003; Stopak et al., 2003). In addition, the Vif-APOBEC complexes were shown to maintain the antiviral activity of APOBEC (Mehle et al., 2004).

Human APOBEC (hAPOBEC) 3G, 3F and 3B inhibit HIV replication and **hAPOBECs 3B and 3C** can inhibit infection by some SIV isolates (Yu et al., 2004). **Murine APOBEC 3** and **rat APOBEC 1** can both inhibit HIV infection when expressed in human cells (Bishop et al., 2004a; Bishop et al., 2004b; Mariani et al., 2003).

Like HIV, MLV packages **hAPOBEC 3G** which inhibits replication of this retrovirus in human cells (Bishop et al., 2004a; Harris et al., 2003; Mangeat et al., 2003). However, murine APOBEC 3 does not inhibit MLV replication (Bishop et al., 2004a). Unlike HIV, MLV is not A-T rich with regard to nucleic acid content so it may not be under 'A' pressure as is the case with HIV (Navarro and Landau, 2004).

1.13.8 Particle maturation

Newly budded virions, whether they are from the cell surface or intracellular vesicles, are immature. The activity of **PR** is required to mature the particle by cleaving **p55 Gag** into **MA, CA, spacer peptide 1 (Sp1), NC, spacer peptide 2 (Sp2)** and **p6**. **Pol** must also be cleaved into **RT, IN** and **PR** for reverse transcription and integration to proceed efficiently in the next round of infection (fig 1.4). Mature, infectious particles of the lentivirus family have cone shaped capsids (figs 1.4 and 1.5) whereas MLV and the other C-type retroviruses have spherical capsid cores. Once matured, the infectious virion may infect the next target cell and the virus life cycle begins anew (fig 1.4, step 8).

1.14 Scope of this thesis

HIV-1 and HIV-2 both infect MØs and T cells. Monocyte derived macrophages (MDM) and PBMC are used to compare the relative infectivity and the replication kinetics of HIV-1 and HIV-2 in these two key infectable cell types. HIV-2 infects PBMC as efficiently as HIV-1, however HIV-2 replicates to much lower levels in MDM. A brief and early burst of replication followed by an inability to spread through MDM culture and an apparent latency of remaining provirus is observed for HIV-2. The HIV-2 V3' loop charge was found to be associated with coreceptor usage but there is only a weak correlation to MØ tropism (chapter 3), so other factors affecting HIV tropism were investigated.

The primary isolate, prCBL 23 was remarkable as being particularly refractory to replication in MDM, and has been shown to be restricted post-entry and post-reverse transcription in these cells (McKnight A, 2001). PrCBL 23 was further shown to be permissive for some cell lines such as U87/CD4/CXCR4 and restricted in HeLa/CD4 cells. This restriction was termed Lv2 (Schmitz et al., 2004). I show that about one-third of HIV-1 and HIV-2 viruses are susceptible to Lv2 restriction (chapter 3) (Marchant et al., 2005).

MCR is a molecular clone of prCBL 23 and the *env* and *gag* genes are shown to act in concert to mediate Lv2 restriction in HeLa/CD4 cells. To explain the involvement of Env in a post-entry event, a compartmentalisation model was developed (Schmitz et al., 2004). I tested this model with hypertonic sucrose inhibition of endocytosis and interruption of lipid rafts to implicate an endocytic lipid raft pathway of restriction for restricted HIV-2 MCR. Dominant negative endocytosis mutants, a more specific tool for studying endocytosis, implicate an Arf6 and dynamin dependent pathway of entry (chapter 4).

Chapter 5 demonstrates that Lv2 is indeed a saturable restriction factor, like Ref1, Fv1 and Lv1, but unlike such restrictions, requires an HIV-2 Env to deliver saturable MLV doses into the restrictive compartment. This chapter compares the saturable nature of Lv2 and other restriction factors with N and B-tropic MLV Gag-Pol cores, expressing various Envs. By classic saturation assays and the development of a variation of this

assay, Lv2 is saturated by both N and B-tropic MLV vector pseudotypes when pseudotyped with HIV-2 Envs.

Similarities between Lv2, Ref1 and Lv1 suggest that tripartite motif protein (TRIM) 5 α or a related protein is responsible for Lv2 restriction. RNA interference (RNAi) is employed in chapter 6 to screen multiple TRIMs for Lv2 activity. TRIMs 1, 18 and 34 are identified as candidates and investigated further. The 3 TRIMs were cloned and over-expressed in permissive cells, rendering these cells restrictive. The TRIM 1 β SPRY/B30.2 domain is implicated as the domain partially responsible for TRIM 1 β restriction activity. Moreover, RNAi knockdown of TRIMs 1, 18 and 34 in HeLa/CD4 cells and challenge with the HIV-2 Env-MLV pseudotypes suggest that other TRIMs, or TRIM like factors, may be Lv2 restrictive (chapter 6).

Chapter 2

Materials and methods

2.1 Buffers and solutions

Table 2.1. Buffers and solutions.

40mM dNTP	10mM dATP; 10mM dCTP; 10mM dGTP; 10mM dTTP.
HEPES-h	10mM HEPES; 1.5mM MgCl ₂ ; 10mM KCl; 0.5mM dithiothreitol.
PBS-A	137mM NaCl; 3mM KCl; 10mM Na ₂ HPO ₄ ; 2mM KH ₂ PO ₄ (pH7.4)
TAE	40mM Tris-HCl pH 7.8; 20mM sodium acetate; 1mM EDTA
TBS	20mM Tris-HCl, pH 7.6, 120mM NaCl
TE	10mM TRIS-HCl, pH 7.4; 1mM EDTA

2.2 Eukaryotic cell culture

Table 2.2. Cell lines used.

Cell line	Original cell type	Reference
293T	Human embryonic fibroblasts	(Graham et al., 1977)
GHOST	Human osteosarcoma	(Cecilia et al., 1998)
H399	Human cervical carcinoma	(Pitcher et al., 1999)
HeLa/CD4	Human cervical carcinoma	(Scherer et al., 1953)
U87/CD4/CCR5	Human glioma	(Deng et al., 1997)
U87/CD4/CXCR4	Human glioma	(Deng et al., 1997)
<i>Mus Dunni</i> tail fibroblasts (MDTF)	Mouse fibroblast	(Lander and Chattopadhyay, 1984)
NP2/CD4/CCR5	Human glioma	(Soda et al., 1999)
NP2/CD4/CXCR4	Human glioma	(Soda et al., 1999)

2.2.1 Passaging cells.

Cells were passaged twice weekly. Adherent cells were detached from tissue culture flasks with 0.5% Trypsin in 0.02% versene then replated in new flasks at a dilution of 1/3 to 1/20 in Dulbecco's modified Eagle's medium (DMEM, Gibco™), 5% fetal calf serum (FCS, Biowest™). Cells expressing CD4 were supplemented with 1 mg/ml G418 (Gibco™) and those expressing CXCR4 or CCR5 were supplemented with 1 µg/ml puromycin.

2.2.2 Freezing cells.

Cells were pelleted at 300g for 5 min and resuspended in DMEM, 20% FCS on ice at approximately 1.0×10^6 cells/ml, diluted 1:1 with a solution of DMEM, 20% FCS, 20% DMSO, 1 ml aliquots were pipetted into cryotubes, placed in a polystyrene container and cooled in a -80°C freezer to freeze slowly overnight (approximately 1°C per min). Frozen cells were transferred to vapour phase liquid nitrogen for long-term storage.

2.2.3 Thawing cells.

Frozen aliquots of cells were thawed from liquid nitrogen storage in a 37°C water bath and added dropwise to DMEM, 10% FCS. Once optimal growth was achieved medium was replaced with normal growth medium and selection antibiotics if applicable (DMEM, 5% FCS).

2.2.4 Preparation of peripheral blood mononuclear cells.

For most studies PBMC were prepared from the same donor as the MDM. Cells were resuspended to 1.0×10^6 cells/ml in RPMI 1640, 10% FCS and 0.5 µg/ml phytohemagglutinin (PHA). Two days later the PBMC were washed twice in serum free RPMI 1640 and then suspended in RPMI 1640, 10% FCS, 20 units (U) /ml of interleukin-2 (IL-2) for two days so that T cells would be selectively propagated.

2.2.5 Preparation of monocyte derived macrophages.

MDM were isolated from peripheral blood monocytes by adherence to plastic as described previously (Simmons et al., 1995), except, washes were performed with serum free RPMI 1640 Penicillin (100 U/ml) and Streptomycin (100 µg/ml). Briefly, buffy coats (Brentwood Blood Transfusion Centre, UK) were diluted 1:1 with PBS. The blood-PBS mixture was then layered onto Lymphoprep™ and centrifuged for 30 min at 700g. The white blood cell layer was harvested, washed twice in PBS, and then suspended in RPMI 1640. Cells were counted, and then plated at 1.0×10^8 cells per 140 mm bacterial dish in 5% heat inactivated human serum (HS) RPMI 1640. After 2 hr, the plates were washed three times in RPMI 1640 and then left to incubate at 37°C overnight. The cells were incubated overnight before washing three times and left to differentiate into MDM for 7 days. After 7 days the MDM were washed 3 times in RPMI 1640, incubated in PBS, 0.01% EDTA at 37°C, and removed by scraping gently with a cell scraper. Harvested cells were pelleted and washed twice, counted, and replated at 1×10^6 , 2×10^5 , 1×10^5 , and 1×10^4 cells per 6, 24, 48, or 96 well tray, respectively.

2.2.6 Transfection of DNA into Eukaryotic cells.

FuGene®-6 was used to transfect DNA into cells as per manufacturers instructions. This reagent provided high transfection efficiency (70 to 90%) for both single and multiple plasmid transfections. Briefly, 16-18 µl of FuGene®-6 per tissue culture tray was added to 500 µl of Optimem® (Gibco™). The Fugene®-6 mixture was added to 3 µg of DNA (per tissue culture plate), pipetted up and down and incubated for 15 min at room temperature. Fresh media (DMEM, 5% FCS) was added to the cells and 10, 20, 42, 80, and 500 µl of the FuGene®-6-DNA mixture was added to each well of a 48, 24, 12, 6 well, and 10 cm plate, respectively. The transfection efficiency was best when transfection complexes were added per well, opposed to batch transfection with replating the following day. This was particularly the case with transfection of the dominant negative endocytosis mutants (table 2.3).

Table 2.3. Transfection volumes of dominant negative mutants.

Volume FuGene-6/plate	DNA	Culture dish	Fresh media (per well)	Transfection mix (per well)
18 μ l	3 μ g	48 well	100 μ l	10 μ l
		24 well	200 μ l	20 μ l
		12 well	400 μ l	40 μ l
		6 well	800 μ l	80 μ l
		10 cm dish	8 ml	500 μ l

Table 2.4. Plasmids

Construct	Plasmids	Encode	Reference
Env	pMDG pMP11-MCR _{env} pMP11-MCN _{env}	VSV-G (VSV Env) MCR Env MCN Env	(Yee et al., 1994) (Schmitz et al., 2004) (Schmitz et al., 2004)
Gag-Pol	pCIG3-MLV-N pCIG3-MLV-B pHIT-80-MLV-Mo p8.2-HIV-1	MLV-N Gag-Pol MLV-B Gag-Pol MLV-Mo Gag-Pol HIV-1 Gag-Pol	(Bock et al., 2000) (Bock et al., 2000) (Soneoka et al., 1995) (Zufferey et al., 1997)
Transfer vectors	pCNCG pCNCZ pC(*TRIM)CZ pCSGW	Neo ^R , eGFP Neo ^R , Zeo ^R TRIM 1, 18, 34 Zeo ^R eGFP	(Soneoka et al., 1995) Keith Aubin Keith Aubin (Demaison et al., 2002)
Cloning vectors	pGEM-T easy-(HIV-2 env V3 loops)	TA cloning vector for cloning and sequencing HIV-2 V3 loops.	Promega Madison, WI, USA
Dominant negative endocytosis mutants	pEGFP-C1-DPF pEGFP-dnCav1 pSR α -Arf6(T27N) pSR α -Dyn(K44A)	eGFP-Eps15-HA (Kan ^R) eGFP-dnCav 1 (Kan ^R) Arf6T27N-HA (Amp ^R) Dynamin K44A-HA (Amp ^R)	(Benmerah et al., 1998) (Pelkmans et al., 2001) (Peters et al., 1995) (van der Bliek 1993)
Infectious molecular clones of HIV	HIV-1 89.6 HIV-1 NL4.3 HIV-2 MCR HIV-2 MCN HIV-2 MCR, MCN-gag+env swaps	HIV-1 89.6 HIV-1 NL4.3 HIV-2 MCR HIV-2 MCN HIV-2 MCR, MCN-gag+env swaps	(Collman et al., 1992) (Adachi et al., 1986) (Schmitz et al., 2004) (Schmitz et al., 2004) (Schmitz et al., 2004)

Zeo^R = Zeomycin resistance, Neo^R = Neomycin resistance, dnCav1 = dominant negative caveolin 1, HA = haemagglutinin tag

2.2.7 RNA interference.

To down regulate the endogenous mRNA transcripts of TRIMs 1, 5, 6, 18, 34 and D6 (control) the following short interfering RNAs (siRNAs) were used: TRIM 1 (NM_052817) 5'-aagcgcaacagcgaacuagaa, TRIM 5α 5'-gcucagggagggucaaguuguu, TRIM 6 (NM_001003818) 5'-aacggagacaagugagguuu, TRIM 18 (NM_010797) 5'-aacggcucuaucgccuuuuau, TRIM 34 (NM_030684) 5'-aaguggacguguccaagaaaa and D6 5'-aaggcugccucucugcaaagu.

SiRNAs directed against TRIMs 1, 6, 18, 34, and D6 control were designed by inputting the nucleotide sequence accession number from the NCBI website into the Qiagen siRNA design tool (<http://www1.qiagen.com/Products/GeneSilencing/CustomSiRna/SiRnaDesigner.aspx>). The sequence with the highest score but the least similarity to sequences in other human genes was used.

The huTRIM 5α siRNA sequence was copied from [(Stremlau et al., 2004), sequence 3].

2.2.8 Transfection of short interfering RNA.

Cells were transfected with siRNA oligonucleotides using Oligofectamine™ (Invitrogen™) as per manufacturers instructions. Briefly, cells were plated in 6 well and 10 cm dishes such that they were approximately 30-50% confluent on the day of transfection. Oligonucleotide (20 μM stock) was diluted in Optimem® and mixed gently (table 2.5). Oligofectamine was diluted in Optimem®, mixed gently and incubated at room temperature for 10 min. The diluted oligonucleotide and Oligofectamine™ were mixed and incubated for a further 20 min. Fresh growth media (DMEM, 5% FCS) was added to the cells and oligonucleotide-Oligofectamine™ complexes were added to each well (table 2.5). Cells were washed and normal growth media was added the following day or replated into 48 and 24 well trays at 4.0×10^4 and 8.0×10^4 cells per well respectively. Cells were challenged with HIV, MLV vector pseudotypes or assayed for gene knockdown the next day (48 hr post-transfection).

Table 2.5. RNA interference transfection volumes.

Culture dish	Oligonucleotide (μl of 20 μM stock) + Optimem[®] (per well)	Oligofectamine[™] + Optimem[®] (per well)	Vol. of medium per well (fresh growth medium added before complexes).	Transfection vol. (complexes added to each well)
6 well	5 μ l + 80 μ l	2 μ l + 6 μ l	800 μ l	93 μ l
10 cm dish	30 μ l + 525 μ l	10 μ l + 35 μ l	5ml	600 μ l

2.2.9 Production of tripartite motif protein expressing cell lines.

NP2/CD4/CXCR4 and U87/CD4/CXCR4 cells stably expressing TRIM proteins 1, 18 and 34 were made by transduction with an MLV derived retroviral vector (table 2.4, fig 2.1). TRIMs amplified from cDNA were subcloned into the pC(TRIM)CZ MLV vector by Keith Aubin (table 2.4). Vector pseudotypes were made with VSV Env as described in section 2.4.2 'virus production by transfection of 293T' (fig 2.1). Cells were transduced with the vector at a multiplicity of infection (MOI) of approximately 1, and Zeomycin containing selection medium was added 48 hr post-transduction. Stable transductants were infected with HIV after stable growth in Zeomycin selection media for about 3 weeks.

2.3 HIV viruses and viral vectors

2.3.1 Viruses and preparation of virus stocks.

All work with primary isolates and molecular clones of HIV-1 and HIV-2 was performed in a containment level 3 laboratory. Primary HIV-2 and HIV-1 strains used in this thesis were prepared in PHA and IL-2 stimulated PBMC from the peripheral blood of infected individuals as described previously (table 2.6) (Reeves et al., 1999; Simmons G, 1996). Briefly, stocks of HIV primary isolates were prepared from the lowest possible passage stock. PBMC from 2 donors were mixed and 5.0×10^6 cells were incubated with 1 ml of virus stock at 37°C for 3 hr. Cell-virus mixture was mixed periodically during incubation. Cells were reconstituted in 5 to 10 ml RPMI 1640, 10%

FCS, supplemented with IL-2. To boost virus stock volume 3 to 5 times the original number of PBMC were added to the infected cells at the peak of infectious virus production, spun at 300g for 5 min and incubated again at 37°C for 3 hr. At the peak of virus production infected cells were spun-down at 500g for 10 min to clarify cell free virus. Aliquots of 0.5 to 1.0 ml were snap frozen in liquid nitrogen and stored in vapour phase liquid nitrogen.

Table 2.6. HIV isolates.

Virus	Coreceptor(s) CCR5 (R5), CXCR4 (X4)	History	Source	Clade	Disease status	Country of origin
HIV-2						
MLC	R5	PI	PBMC	A	ARC	Portugal
TER	R5+ R1, R3, CXCR6	PI	PBMC	A	AIDS	Portugal
ALI	R5+ R1	PI	PBMC	A	ARC	Portugal
JAU	R5/X4+ R1, R2b, R3, R8	PI	PBMC	A	AIDS	Portugal
ST	R5/X4+ R1, R2b, R3	PI	PBMC	A	AIDS	Senegal
ETP	R5/X4+ R1, R2b, R3	PI	PBMC	A	AIDS	Portugal
MIR	R5/X4+ R1, R2b, R3	PI	PBMC	A	AIDS	Guinea-Bissau
PrCBL-20	R5/X4+ R1, R2b, R3	PI	PBMC	A	AIDS	The Gambia
PrCBL-23 (MCR)	R5/X4+ R1, R2b, R3	PI / (MC)	PBMC	A	AIDS	The Gambia
MIL	X4	PI	PBMC	A	AIDS	Portugal
SAB	X4+ R3	PI	PBMC	A	AIDS	Portugal
AND	X4+ R1, R2b, R3	PI	PBMC	A	AIDS	Portugal
CBL-23 (MCN)	R5/X4	TCLA / (MC)	H9	A	AIDS	Portugal
Rod (ACR 23)	X4 + R3	TCLA / (MC)	H9	A	AIDS	Senegal
HIV-1						
SF162	R5	PI / MC	Brain	B	AIDS	USA
SL-2	R5	PI	PBMC	B	Asymptomatic	Thailand
SF-2	R5/X4	TCLA		B		USA
2006	X4	PI	PBMC	NK	AIDS	UK
2076	R5/X4+ R3, R8, STRL-33, GPR-15	PI	PBMC	B	AIDS	UK
2026	R5/X4+ R3, R8, STRL-33, GPR-15	PI	PBMC	B	AIDS	UK
2044	X4	PI	PBMC	B	AIDS	UK
NL4.3	X4	TCLA / MC	PBMC	B	AIDS	USA/France
89.6	R5/X4	TCLA / MC	PBMC	B	AIDS	USA
RF	X4	TCLA	PBMC	B	AIDS	Haiti
M13	R5/X4		PBMC	B	AIDS	USA
HAN-2	X4	PI	PBMC	B	AIDS	Germany
IIIB	X4	TCLA	PBMC	B	AIDS	France
MN	X4	TCLA	PBMC	B	AIDS	USA

'+' denotes additional coreceptor use as determined previously (Reeves et al, 1999; McKnight et al, 1998; Neil et al, 2005), NK=not known, Coreceptor shown in bold denotes predominant use of that coreceptor. R1 = CCR1, R2b = CCR2b, R3 = CCR3, R8 = CCR8. TCLA = T-cell line adapted, PI = primary isolate, MC = molecular clone, ARC = AIDS related complex.

2.3.2 Virus production by transfection of 293T cells.

HIV molecular clone stocks were prepared in 293T cells in 10 cm culture dish by transfection with FuGene[®]-6 (table 2.4), as described in 'Transfection of DNA into eukaryotic cells' except for some changes: the day after transfection, 8 ml of transfection media was replaced with 14 ml of normal growth media in a 10 cm dish to boost stock volume. This was replaced the following day with a further 14 ml and the virus stocks were then harvested and aliquoted into 0.5 ml 72 hr post-transfection. Harvests of equal infectivity could be taken at 48, 72 and 96 hr post-transfection. The 293T cells were cultured in GlutaMAX[™] supplemented DMEM (Gibco[™]) and 10% FCS in a 37°C and 10% CO₂ incubator.

2.3.3 N-tropic, B-tropic and Moloney MLV virion pseudotyping.

VSV-enveloped, MLV-N/B-Gag-Pol particles were produced from a combination of three plasmids as described previously (fig 2.1, table 2.4) (Towers et al., 2000). MLV-N, B and Moloney (Mo) Gag-Pol cores (packaging constructs) were expressed from pCIG3-N, pCIG3-B and pHIT60 respectively, and pseudotyped with VSV-G expressed from pMDG, or MCR and MCN Env from pSVIII (fig 2.1, table 2.4). The proviral reporter genome pCNCG encoding eGFP was packaged into the pseudovirions (fig 2.1, table 2.4). Three µg pCNCG, 2 µg pCIG3-N or B, and 1 µg pMDG were mixed and a confluent layer of 293T cells in a 10 cm dish was transfected in the presence of 18ul FuGene[®]-6 (see 'transfection of DNA into eukaryotic cells'). Pseudotype particles were harvested 48, 72 and 96 hr post-transfection and viral titres were determined on MDTF and by RT-ELISA.

2.3.4 HIV-1 vector pseudotypes.

HIV-1-based vectors were derived from the packaging construct p8.91 (Zufferey et al., 1997), and the eGFP encoding vector genome pCSGW (table 2.4) (Demaision et al., 2002), in conjunction with pMP11, encoding either MCN or MCR Env (Schmitz et al., 2004). Molecular clones, pseudotyped viruses, and vectors were produced by transient transfection of 293T cells, as described above in 'N and B-tropic and Moloney MLV virion pseudotyping' and fig 2.1.

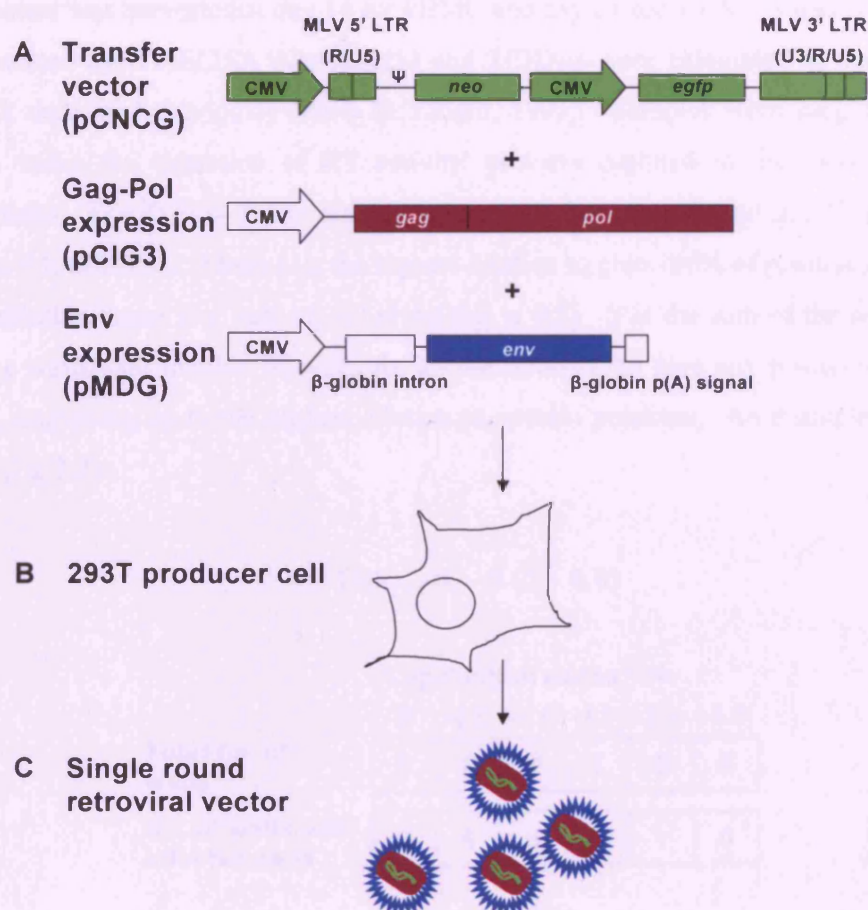


Figure 2.1. Production of retroviral vector by triple transfection.

(A) Transfer vector, Gag-Pol and Env expression plasmids were mixed and transfected into (B) 293T producer cells. (C) The only construct to contain the packaging signal (ψ) is the transfer vector (devoid of genes required for replication) so it is preferentially packaged and the virions that are produced are single round only.

2.4 Infectivity assays

2.4.1 Tissue culture infectious dose 50.

Tissue culture infectious dose 50s (TCID₅₀) of HIV-1 and HIV-2 were estimated on both MDM and PBMC. MDM and PBMC were plated on 96 well trays at 10^4 and 10^5 cells per well, respectively. Virus was serially diluted in half logs and 50 μ l dilutions were incubated with either PBMC or MDMs. After overnight incubation with virus inoculum cells were washed 3 times in growth media which was changed every 7 days.

Supernatant was harvested at day 14 for PBMC and day 21 for MDM. Virus production was detected by RT-ELISA (Caviditech) and TCID₅₀s were calculated by the Karber method, described previously (Aine McKnight, 1995). Samples were subject to RT-ELISA using the 'detection of RT activity' protocol outlined in the manufacturers instructions. The TCID₅₀ is the dilution where cells become infected in 50% of wells: $TCID_{50} = L - d(S - 0.5)$. Where L is the highest dilution to give 100% of positives, while d is the dilution factor (for half log dilutions this is 0.5). S is the sum of the number of positive wells/total number of wells for all the dilutions to give any positives starting from L and going up to the highest dilution to contain positives. An example is given below (fig 2.2).

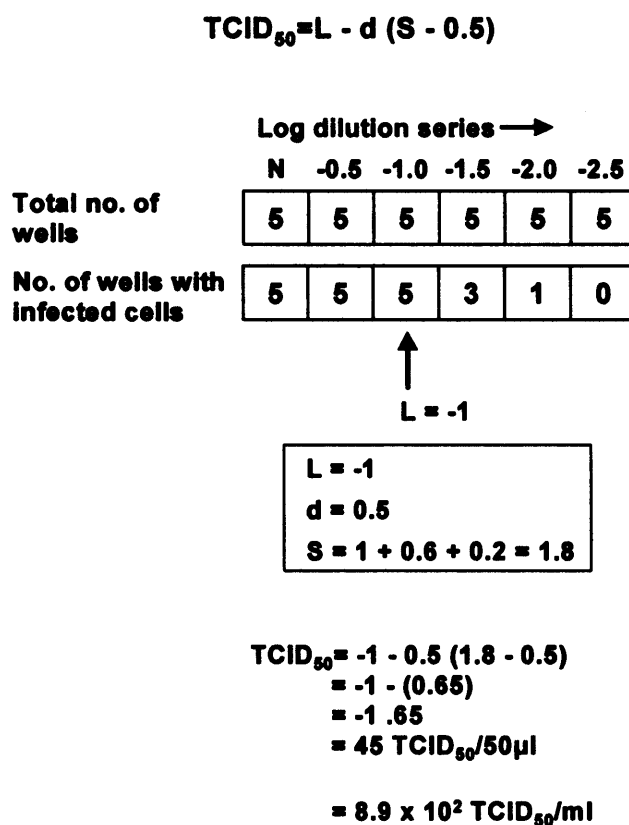


Figure 2.2. Example tissue culture infectious dose 50 calculation.

2.4.2 Monocyte derived macrophage time courses of infection.

MDM time-courses were done in 6-well trays. Cells were plated at 1.5×10^6 cells per well. The following day, 1ml of 10^4 TCID₅₀/ml (as determined on PBMC) of virus diluted in RPMI 1640, 10% heat-inactivated HS was added to its corresponding well. After 3 hr of infection, the virus was removed and the cells were washed with serum free RPMI 1640. Fresh 10% HS in RPMI 1640 was added and the first aliquot, time 0, was taken. Every two days, an aliquot was removed and stored at -40°C for determination of RT activity and focus forming units (FFUs). After the removal of each time-point aliquot the MDM were washed once with 10% heat-inactivated HS RPMI 1640 to eliminate the possibility of measuring carry-over virus in the following time-point.

2.4.3 Cholesterol depletion.

To aid cell adherence during cholesterol depletion, tissue culture surfaces were treated with 1 part 2% bovine gelatine (Sigma) to 1 part serum free DMEM for 1 hr at room temp. Cells were plated the night before they were needed on 48 well trays at approximately 5.0×10^4 cells per well and in 75 cm² medium flasks at 1.0×10^7 cells/flask. The best results were obtained when four confluent-medium 75 cm² flasks were split into eight gelatine-coated-medium flasks and fractionated 3 days later.

Prior to virus addition, cells were washed with serum free media then treated with 10 mM methyl- β -cyclodextrin (M β CD) for 20 min at 37°C . The cells were rinsed once with serum free media, virus dilutions were added and allowed to bind to the cells on ice for 1 hr. After synchronisation on ice, virus inoculum was removed and serum free medium, with or without water-soluble cholesterol, was added to the cells, and transferred to a 37°C incubator to allow infection to proceed for 2 hr. Normal growth media was added to the cells, fixed and stained for HIV infection 3 days later.

2.4.4 Inhibition of endocytosis with hypertonic sucrose.

Cells were plated at subconfluence, as described for 'cholesterol depletion', the day before they were needed. The cells were washed once with growth media, virus dilutions were added, and placed on ice for 1 hr. Normal growth media (DMEM, 5%

FCS) was replaced with 0.45 M sucrose, DMEM, 5% FCS for 50 min in a 37°C incubator. The sucrose media was then removed and replaced with normal growth media. The cells were fixed and stained 3 days later for HIV infection.

To control for possible mitosis effects of hypertonic sucrose the cells were pre-treated with sucrose for 20 min prior to addition of virus.

2.4.5 Transfection of dominant-negative mutants.

HeLa/CD4, U87/CD4/CXCR4, and H399 cells were plated the night before. Cells were plated on gelatin-coated plates to promote adherence and time courses for optimal transfection expression were conducted to minimise duration of the experiment. The day of transfection, growth medium was replaced with DMEM, 5% FCS and transfections were carried out overnight. The cells were washed once and transfection medium was replaced with normal growth medium the following day. Cells recovered in normal growth medium for at least 1 day post-transfection to eliminate any effect the transfection reagent might have on HIV infection (transfection reagents relieve Lv2 restriction). Transfection and eGFP expression also proved to be toxic since the eGFP control caused extensive cell death in some cases: DNA and FuGene[®]-6 were titrated onto cells to ascertain the optimal DNA and FuGene[®]-6 concentrations. Cells were infected, 48 hr post-transfection, with HIV-1 and HIV-2 on ice for 1 hr, incubated at 37°C, media was replaced with normal growth media 4 hr later and cells were fixed and stained for HIV infection 2 days later (see also table 2.3 and 'transfection of eukaryotic cells').

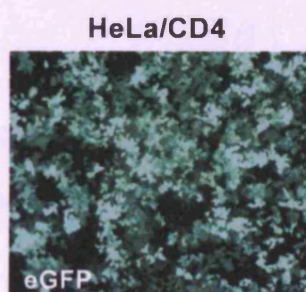


Figure 2.3. Transfection efficiency of dominant negative mutant constructs.

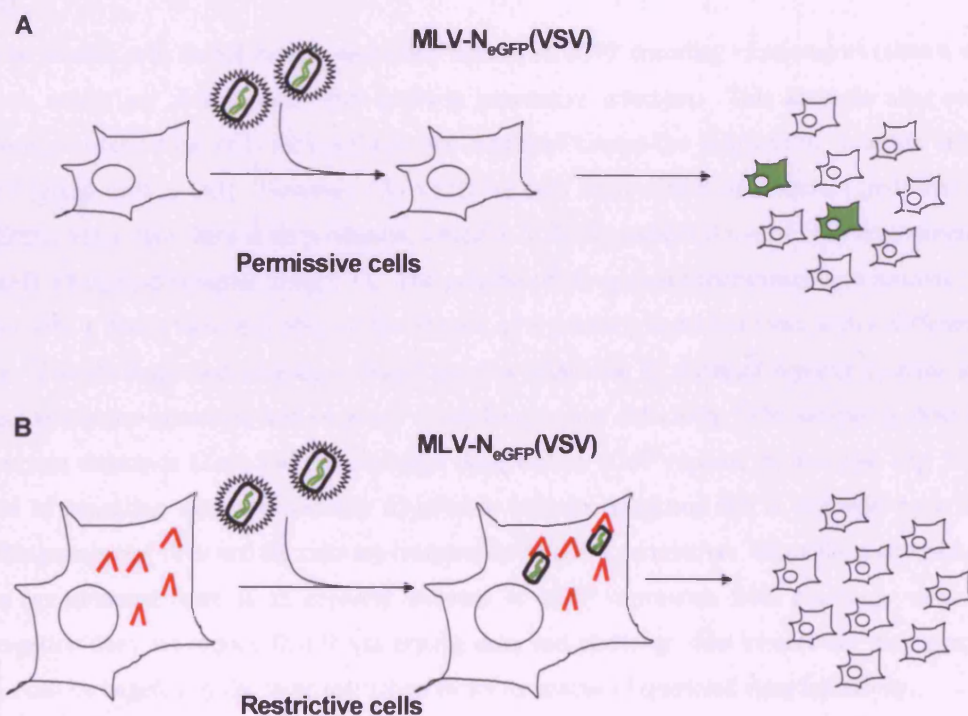
An eGFP expressing plasmid (pCNCG) was used as a transfection control in all dominant negative mutant experiments in chapter 4 and transfection efficiency was approximately 100% in HeLa/CD4 cells.

Cells were transfected with FuGene® (Roche) as described above, and visualised by blue light before staining for HIV infection.

2.4.6 Abrogation assays

2.4.6.1 Ref1 saturation.

Ref1 abrogation control was conducted as described previously (fig 2.4c) (Schmitz et al., 2004; Towers et al., 2002) and in results chapter 5 (fig 5.1). HeLa/CD4, U87/CD4/CXCR4, and HOS/CD4/CXCR4 cells were plated in 48 well trays at 5×10^3 cells per well 24 hr prior to infection. Cells were treated with high doses of MLV-N_{Zeo}(VSV) vector pseudotype [approximately 10^5 IU/ml as determined on MDTF cells or 10^4 pg/ml RT activity (determined by RT ELISA)] for 6 hr. Cells were washed with normal growth media and MLV-N_{eGFP}(VSV) was titrated on the cells and eGFP transduced cells were detected by flow cytometry 3 days later.



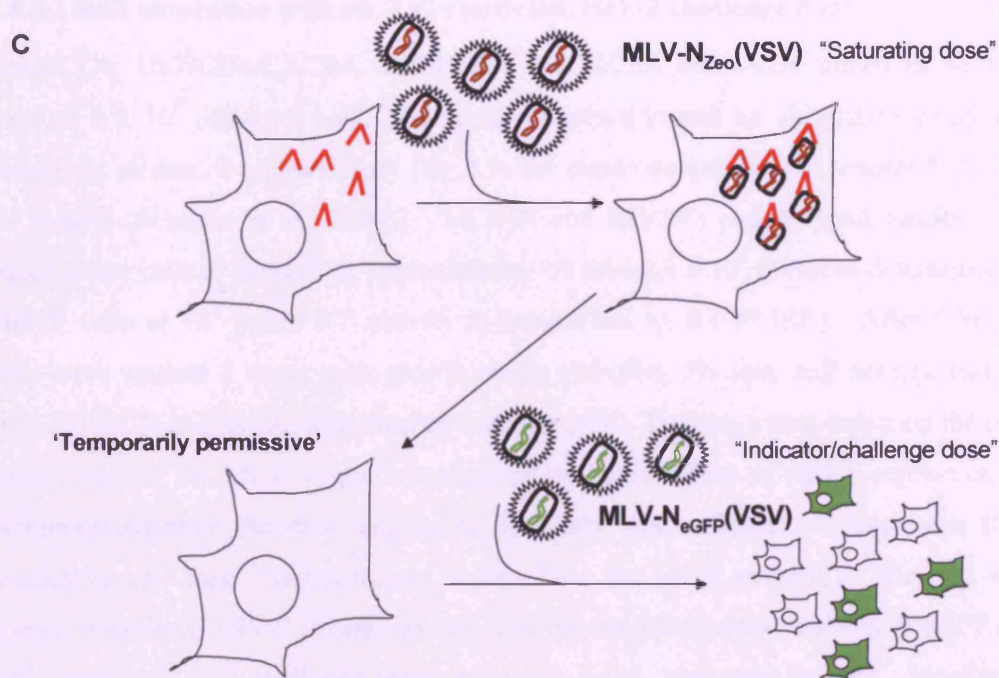


Figure 2.4. Abrogation of restriction factors in restrictive versus permissive cells: Ref1.

(A) Permissive cells do not express restriction factors, all eGFP encoding virus vectors (shown with green nucleic acids) are able to enter and establish productive infection. This scenario also occurs with challenge of restrictive cells with resistant/non-restricted viruses (no restriction). Infected cells express eGFP [green cells in (A)]. However, (B) restrictive cells express restriction factors (red) that inhibit all challenge virus, thus there is no productive infection in this hypothetical model. (C) Experimental model of Ref1 abrogation (chapter 5, fig 5.1). The purpose of abrogation experiments is to saturate restriction factor with a decoy virus and observe the 'rescue' of a similarly restricted virus with a different reporter gene. The challenge and saturating viruses must be detectable by different reporter systems in order to detect restriction-saturation and recovery of challenge virus infectivity [The saturating dose conferred Zeomycin resistance (Zeo) and the challenge dose was an eGFP reporter in this case (fig 5.1)]. High doses of saturating virus are normally required to saturate restriction that is followed by a titration of challenge/reporter virus and the cells are temporarily rendered permissive. When the restrictive factor has been pre-saturated there is an apparent increase in eGFP expression from challenge virus infection. Abrogation does not require that the saturating dose and challenge dose viruses are the same, however they must be targeted by the same restriction factor for rescue of restricted virus infectivity.

2.4.6.2 Ref1 saturation with an, Lv2 restricted, HIV-2 challenge dose.

HeLa/CD4, U87/CD4/CXCR4, and HOS/CD4/CXCR4 cells were plated in 48 well trays at 5×10^3 cells per well 24 hr prior to infection and an abrogation assay was conducted as described previously [fig 2.5a for experimental design; chapter 5, fig 5.3 for results (Schmitz et al., 2004)]. MLV-N and B(VSV) pseudotyped viruses were added to the cells at an MOI of approximately 10 (about 1×10^5 IU/ml as determined on MDTF cells or 10^4 pg/ml RT activity as determined by RT-ELISA). After 6 hr, the cells were washed 3 times with growth media (DMEM, 5% fetal calf serum), and the HIV-2s, MCN and MCR, were titrated onto the cells. Two days post-infection the cells were screened for MLV vector transduction by visualisation of eGFP expression, to determine whether the dose applied to the cells was sufficient to overcome Ref1 restriction, and then immunostained 3 days later for MCN and MCR infection with human polyclonal HIV-2 serum. In this case the saturating dose read-out is eGFP (fig 2.5b) and the HIV-2 challenge virus reporter is β -Gal, blue spot staining. Monitoring eGFP expression was a convenient control to determine whether the MLV-N_{eGFP} dose delivered was a Ref1 saturating one (fig 2.5b).

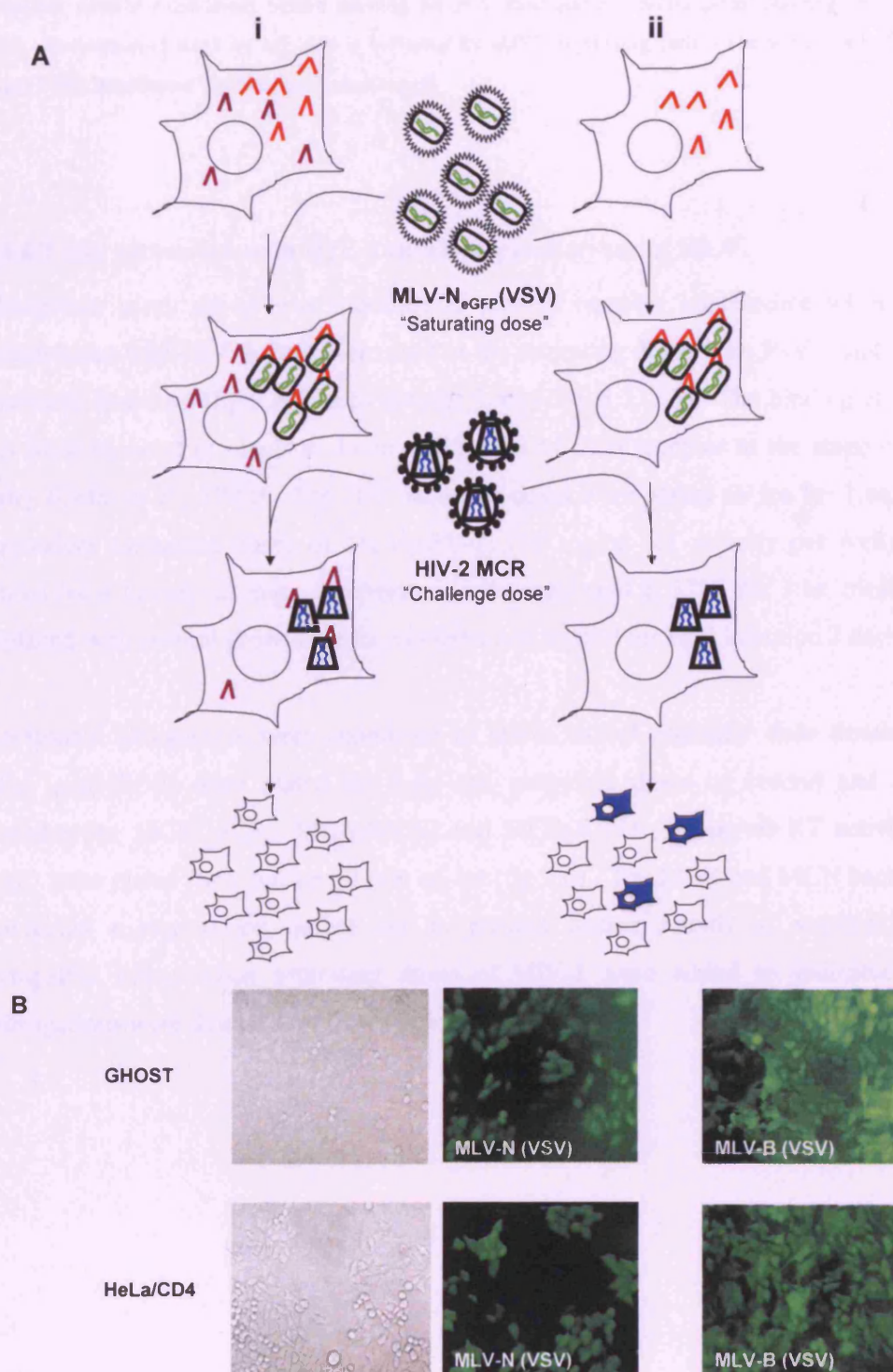


Figure 2.5. Determination of relationship between Ref1 and Lv2.

(A) The experimental model for determining whether Lv2 (purple) and Ref1 (red) are the same restriction factor. (i) If Lv2 and Ref1 are different restriction factors there would be no expected recovery of Lv2 by Ref1 saturation. (ii) If Ref1 and Lv2 were the same restriction factor then there would be recovery of Lv2 restricted HIV-2 MCR with the saturation of Ref1 with MLV-N(VSV) vector pseudotypes. (B) EGFP expression by MLV-N_{eGFP}(VSV) particles in (A) allowed for monitoring of Ref1 saturation during Lv2

saturation control experiment before staining for HIV infection by in-situ β -Gal staining (blue spots, FFU). Saturation of Ref1 by MLV-N is indicated by eGFP expressing cells. The same dose of MLV-B_{eGFP}(VSV) transduced 100% of cells challenged.

2.4.6.3 Lv2 saturation with HIV-2 envelope pseudotypes of MLV.

Abrogation assay set-up was modified to prevent receptor interference when MLV pseudotypes with HIV-2 Envs were used as the saturating doses with HIV-1 and HIV-2 challenge titrations (fig 2.6; results chapter 5, figs 5.6, 5.7, 5.8). The binding of gp120 has been reported to result in down modulation of CD4 receptor at the stage of HIV entry (Cefai et al., 1992). The HIV indicator doses were plated on ice for 1 hr, later, equivalent saturating doses of MLV(HIV-2) (10^4 pg/ml RT activity per well) were added for a further 30 min. Abrogations were incubated at 37°C for 3 hr, media was replaced with normal growth media and fixed and stained for HIV infection 3 days later.

Reciprocal abrogations were conducted as above except indicator dose titrations of MLV_{eGFP}(HIV-2) were plated for 1 hr and saturating doses of control and HIV-2 pseudotypes MCR(MCR), MCN(MCN) and MCR(VSV) (10^4 pg/ml RT activity per well) were plated for a further 30 min on ice (fig 2.6). The MCR and MCN backbones contained a stop codon before *env* to prevent further rounds of replication and cytopathic effect when saturating doses of HIV-2 were added to indicator cells. Abrogations were detected by flow cytometry 3 days later.

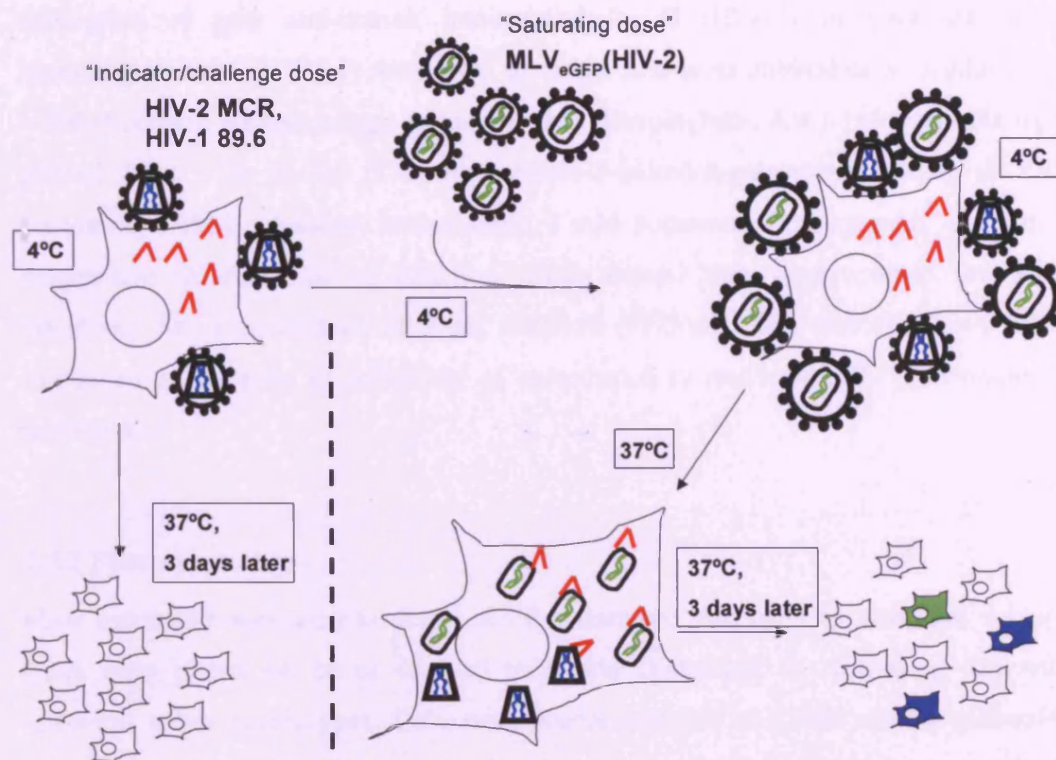


Figure 2.6. Abrogation experiment design for preventing receptor interference by saturating doses of HIV-2 envelope pseudotypes of MLV.

To prevent down regulation of CD4 receptor by MLV vectors with HIV-2 Envs both the challenge HIV-1 and HIV-2, and the saturating MLV(HIV-2) virus doses were bound to the target cells on ice. The challenge virus titrations were plated first to prevent occlusion of available receptor by saturating MLV(HIV-2) viruses. Dashed line separates the different outcomes (right) with and (left) without MLV(HIV-2) saturation (chapter 5; fig 5.6, 5.7, 5.8). Lv2 restriction factors are shown in red.

2.5 Detection of infectivity

2.5.1 Immunostaining of HIV-2- and HIV-1-infected cells and calculation of restriction.

The immunostaining method has been described previously (Schmitz et al., 2004). Briefly, methanol-acetone (1:1)-fixed cells infected with HIV-2 or HIV-1 were immunostained with HIV-2 HS diluted 1/4,000 or anti-HIV-1 p24 monoclonal antibody diluted 1/100 (1:1 mix of EVA 365 and 366 from the Medical Research Council AIDS Reagent Program, Potters Bar, United Kingdom). Second-layer β -galactosidase

conjugates of goat anti-human immunoglobulin G (HIV-2) or goat anti-mouse immunoglobulin G (HIV-1) were used to detect first-layer antibodies at a dilution of 1:400 (Southern Biotechnology Associates, Inc., Birmingham, Ala.). Infected cells were stained blue with X-Gal (5-bromo-4-chloro-3-indoyl- β -galactopyranoside) in PBS containing 3 mM potassium ferri-cyanide, 3 mM potassium ferro-cyanide, and 1 mM magnesium chloride. Foci of infection, which stained blue, were counted, and virus infectivity was estimated as FFU per millilitre (FFU/ml). The restriction (n -fold) is calculated as the ratio of infectivity of unrestricted to restricted cells (unrestricted \div restricted).

2.5.2 Flow cytometry.

Flow cytometry was used to detect eGFP transduced into cells by retroviral vectors. Cells were plated on 24 or 48 well trays and challenged the following day with retroviral vector pseudotypes. Cells were washed with 200 μ l 1:5000 versene (Gibco™) 3 to 5 days post-transduction, detached with a further 200 μ l 1:5000 versene, and fixed with 200 μ l 3.8% formol saline for 20 min at room temperature. At this stage cells were deemed safe to remove from the containment level 3 suite and detect eGFP on a Becton Dickinson FACScan flow cytometer at containment level 1.

2.5.3 Reverse transcriptase enzyme linked immunosorbent assay.

RT ELISA kits were purchased from Caviditech. ELISA plates (96 well) are prebound with poly-rA and an oligo-dT primer. The poly-rA serves as a template for the sample virus RT to build the new DNA strand with bromo-deoxyuridine-triphosphate (BrdU). An anti-BrdU mAb conjugated to alkaline phosphatase (AP) detects the nascent BrdU strand. This is a colorimetric assay that detects the AP cleavage of para-nitro-phenyl phosphate at 405 nm.

2.5.4 Detection of infectivity by *gag*-LTR polymerase chain reaction (see polymerase chain reaction).

2.6 Polymerase chain reaction

2.6.1 Polymerase chain reaction of HIV-2 sequences from HIV-2 infected macrophages.

For *gag*-LTR and ERV-3 polymerase chain reaction (PCR), MDM were set-up as for MDM time-courses (described in 'infectivity assays') except the viruses were treated with 25 U DNase (Roche) per 0.5ml vial of virus before dilution and incubation with cells. At the appropriate time-point the infected or negative control uninfected MDM were washed once with versene 1:5000, incubated for 10 min in versene 1:5000 at 37°C, and harvested by scraping. The mixture was centrifuged at 4000g for 5 min, the supernatant was removed, and the cell pellet was stored at -20°C.

DNA was prepared from harvested MDM with the Qia-amp Blood and Tissue Mini Kit (Qiagen[®]). Briefly, cells were spun and resuspended in 200µl of PBS. After addition of Qia-amp lysis buffer and proteinase K the cells were digested at 56°C for 3 hr. DNA was ethanol precipitated and washed twice on a Qia-amp column, and then eluted in 100µl of distilled water.

2.6.2 *gag*-LTR polymerase chain reaction.

PCR reactions were run using 5 µl of DNA from a DNA extraction of 1.5×10^6 MDM (approximately 250 ng of cellular DNA). The 5' *gag*-LTR primer 2713 (5'-TCTCTCCAGCACTAGCAGGTAGAG) is complementary to a sequence in 'R' of the HIV-2 LTR. The 3' *gag*-LTR, 2715 (5'-CAAGACGGAGTTTCTCGCGCCCAT) primer is complementary to a sequence within HIV-2 *gag*. The *gag*-LTR primers measure full-length transcripts of reverse transcription and integrated HIV-2 provirus (529-bp fragment). The 5' and 3' ERV-3 primers detect sequences within the *env* region of the human endogenous retrovirus ERV-3 (ERV-3 forward 5'-GAGGCATAACTATAGGAGATTGG, ERV-3 reverse 5'-CCTTTCCAAGTCTGAACTG; 404bp fragment). There is one copy of ERV-3 per haploid human genome and therefore ERV-3 PCR acts as a cellular DNA input control (Cohen et al., 1985; O'Connell et al., 1984). The *gag*-LTR reactions were carried out with the Roche High-Fidelity PCR system. For a 50 µl reaction the concentrations of

the reaction components were as follows: buffer 3 (undisclosed concentration of DMSO, Tween-20, and Nonidet-P40 to minimise secondary structure) containing 2.75 mM final concentration $MgCl_2$, 1 pmol of each primer, dNTPs were added to a final concentration of 4 mM, and 1.75 U of *Taq* polymerase per reaction. Roche buffer 3 was used to minimise template secondary structure and aid in the amplification of viral reverse transcription products. Conditions for *gag*-LTR PCR were as follows: 4 min at 94°C, followed by 35 cycles of 94°C for 1 min, 65°C for 1 min, 68°C for 1.5 min. Conditions for ERV-3 PCR were as follows: 4 min at 94°C, followed by 35 cycles of 94°C for 45 s, 60°C for 45 s, 68°C for 45 s. PCR products were run on a 2% agarose gel and viewed under UV. The *gag*-LTR PCR products ran at approximately 0.5 kbp and the ERV-3 PCR products ran at approximately 0.4 kbp. An elongation temperature of 68°C was chosen because this is the optimal temperature for the *Taq* Extender proofreading activity.

2.6.3 GAPDH polymerase chain reaction.

The forward (5'-TGGTGAAGACGCCAGTGG) and reverse (5'-ATGGGGAAGGTGAAGGTC) GAPDH primers were used to detect equal cDNA input for RT-PCR (below). The annealing conditions were as follows: 4 min at 94°C, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, 74°C for 30 s.

2.6.4 Reverse transcriptase polymerase chain reaction.

RT-PCR to measure *gag*-LTR RNA transcripts was conducted on MDM infected with HIV-2 JAU and HIV-1 SF162. MDM in 6-well trays were incubated with equivalent input (5.0×10^3 FFU) of either JAU or SF162 for 3 hr, time points were harvested every 24 hr up to and including 5 days post-infection. The infected MDM were harvested with Trizol[®] RNA extraction reagent and the cDNA was prepared. Cells for RT-PCR were washed once in PBS and dissolved in-situ with 1ml of Trizol[®] (Gibco-BRL) RNA extraction reagent. RNA was extracted as per manufacturers instructions. Briefly, extracted RNA was quantified spectrophotometrically by measuring the absorbance at 260 nm. One μ g of total RNA was DNase treated for 30 min at 37°C. To remove RNA secondary structure and to inactivate the DNase, two fifths of the DNase treated RNA was heated at 75°C for 10 min in the presence of DNase stop solution and random

primers, then put on ice. The RNA was added to a mixture that was either RT positive or negative (to control for contaminating DNA). RT reactions, plus or minus RT, were incubated at the RT reaction temperature of 42°C for 1 hr. Two µl of each RT reaction was put into a 50 µl GAPDH and *gag*-LTR PCR reaction and subject to the PCR conditions as mentioned in 'polymerase chain reaction'.

2.6.5 Quantitative polymerase chain reaction.

U87/CD4/CXCR4, H399, and HeLa/CD4 cells were seeded in 24 well trays at 5 to 8 x 10⁴ cells per well and treated with 5.0 x 10³ FFU (determined on U87/CD4/CXCR4) of either MCN or MCR pre-treated with DNaseI (Roche) for 1 h at 37°C. 100 ng of total DNA was used for qPCR with primers: For HIV-2 *gag*-LTR: 900 nM forward primer (5'-CGGCTGAGTGAAGGCAGTAAG-3'), 300 nM reverse primer (5'-TTGGCCTGCGCCTTTCTA-3'), 150 nM *taqman* probe (5'-6-carboxyfluorescein-5'-CAGGAACAAACCACGACGGAGTGCTC-3'-6-carboxytetramethyl-rhodamine-3').

For HIV-1 packaging signal (Ψ), 300 nM forward primer (TGGGCAAGCAGGGAGCTA), 300 nM reverse (TCCTGTCTGAAGGGATGGTTGT) and 150 nM *Taqman* probe (5'-6-carboxyfluorescein-AACGATTCGCAGTTAATCCTGGCCTGTT-6-carboxytetramethyl-rhodamine-3').

First strand synthesis of the MLV pCNCG was monitored by amplification of the vector eGFP cDNA sequence: 300 nM forward primer (5'-CAACAGCCACAACGTCTATATCAT-3'), 300 nM reverse (5'-ATGTTGTGGCGGATCTTGAAG-3') and 150 nM *taqman* probe (5'-6-carboxyfluorescein-CCGACAAGCAGAAGAACGGCATCAA-6-carboxytetramethyl-rhodamine-3'). The PCR conditions consisted of one cycle of denaturation (95°C for 10 min) followed by 45 cycles of amplification (95°C for 15 s, 60°C for 1 min). The amplification, data acquisition, and analysis were performed using the ABI PRISM 7000 sequence detection system.

2.7 Cloning of the HIV-2 envelope third variable loop region

2.7.1 Cloning and sequencing of the HIV-2 third variable loop.

3-5 x 10⁶ PBMC were infected with HIV-2 virus stocks. Three days after infection the infected cells were harvested and DNA extracted with the DNA Blood Mini Kit (Qiagen). V3 loops were amplified with primers F13418 (5'-GGTTTGGCTTTAATGGCACTAGAG) and F13420 (5'-TTCTCCTCTGCAGTTAGTCCACAT) in the HIV-2 V3 region (243-bp fragment). The amplified product was purified from ethidium bromide-containing 1% agarose gel with the Qiagen Agarose Gel Extraction kit. The V3 region was cloned into pGEM-T easy with the pGEM-T easy TA cloning kit (Promega, Madison, WI, USA). Clones were screened and sequenced with primers F13418 and F13420. Sequencing was carried out with the CEQ-DTCS Beckman Coulter Quick Start kit (Beckman-Coulter, Fullerton, CA, USA). The V3 loops from HIV-2 isolates were sequenced from at least 3 clones or directly from PCR product.

Direct sequencing of PCR reactions of the HIV-2 isolates was used to confirm the cloned sequences. Briefly, the V3 region was amplified with primers F13418 and F13420, the product was analysed on an ethidium bromide-containing 1% agarose gel, extracted with the Qiagen PCR extraction kit, and then sequenced (see 'Cloning and sequencing of V3 loops' above).

V3 loop sequences were aligned using the Clustal nucleic acid sequence alignment program.

The net charge of each of the V3 loops was estimated from the amino acid sequence by totalling the number of positively charged Arginine, Lysine, and Histidine residues and then subtracting the number of negatively charged Aspartate and Glutamate residues.

2.7.2 Sequencing sample preparation for Beckman CEQ-2000.

Mini-prep DNA was prepared for sequencing by the dye-labelled-dideoxy-nucleotide chain termination method. For each sequencing reaction 6 µl of premix was added to 5 pmol of primer (T7 and SP6 of pGEM-T easy or F13418 and F13420) and 1 µl of a 50 µl plasmid miniprep and made up to a total of 10 µl with water. Reactions were run on

an MJ PCR thermocycler for 24 cycles at the following conditions: 96°C for 30 s, 50°C for 15 s, 60°C for 4 min, and finished at 4°C.

Sequencing samples were cleaned-up after the labelling reaction. Fifteen µl of premix (2 µl 3 M cold NaOAc, 2 µl 100 mM EDTA, 1 µl glycogen, and 10 µl of water) was added to each sample and mixed. Sixty µl of -20°C 95% ethanol was added to each tube and vortexed. Samples were left on ice for 10 min then centrifuged at room temperature for 15 min to promote the sticking of precipitated DNA to the sample tube. Supernatant was removed and 200 µl -20°C 70% ethanol was added for 10 s to wash pellet. Tubes were left open on bench for 30 min to dry. Pellets were dissolved in deionised formamide. Samples were loaded into sequencing plates and overlayed with a drop of oil.

2.8 Maxi-prep of plasmid stocks

2.8.1 Transformation of bacteria.

HB 101 or TOP F10' bacteria were mixed with plasmid DNA on ice and transformed by heat shock at 42°C for 30 s. Transformed bacteria were plated on LB agar plates supplemented with 100 µg/ml ampicillin for selection of transformants, or 50 µg/ml kanamycin for pEGFP-C1/C2 (Clontech™) plasmids. Colonies were picked and minipreps (Qiagen) were performed on 5 ml overnight cultures of inoculated LB-broth with 100 µg/ml ampicillin.

2.8.2 Restriction enzyme digest of plasmid stocks.

Restriction enzyme digests were performed on the above minipreps to confirm the identity of transformed plasmid DNA. Digests were performed on 1 µg of total DNA with 10 U of restriction enzyme per reaction, between 37 and 65°C (depending on the restriction enzyme) for 1 hr. Restriction products were run on a 1.5 % agarose gel for 2 hr at 60 V and the digestion pattern was compared to that of a positive control run in parallel (if available) or the predicted pattern.

2.8.3 Hi-speed plasmid maxi kit.

Positive HB 101, TOP F10' or STBL 2 bacteria colonies were repicked and plasmid stocks were grown using LB culture broth at 37°C (or 30°C for STBL 2) overnight. All plasmid-transformed bacteria were grown-up in approximately 150 ml of LB broth, except 250 ml for low copy plasmids [pMDG (VSV-G)], with 100 µg/ml ampicillin or 50 µg/ml kanamycin. Maxi prep was performed as per manufacturers instructions for the Hi-speed plasmid maxi kit (Qiagen). Concentration and quality of plasmid DNA was determined by the absorbance at 260 nm and the 260/280 nm ratio, respectively, on an ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA).

2.9 Subcellular fractionation

2.9.1 Preparation of cell lysates.

The preparation and fractionation of cell lysates were carried out on ice unless otherwise stated. Cells (1.0×10^7 to 5.0×10^7) were harvested with versene 1:5 000 (Gibco™) and then washed twice with PBS. The cell pellet was resuspended in 1 ml hypotonic HEPES-h buffer, Roche EDTA free complete anti-protease pellet, pH 7.9 at 4°C and centrifuged for 5 min at 5000 rpm (500g) and 4°C in a Fischer refrigerated microcentrifuge. 300 µl 1% Triton X-100 HEPES-h buffer was added to the cell pellet, left for 30 min on ice, then homogenised with 10 strokes of a Dounce homogeniser. The homogeniser was washed with 100 µl of 1% Triton X-100 HEPES-h buffer and added to the lysate. To remove nuclei, the lysate was spun at 2000 rpm (300 g) for 3 min and 4°C, then the supernatant containing cell membranes was transferred to a new tube. Nuclei were stored at -20°C for PCR and western blot. The resulting cell lysate was loaded onto a sucrose gradient.

2.9.2 Isolation of lipid rafts by sucrose gradient fractionation.

Four hundred µl of cell lysate was mixed with 800 µl 60% sucrose in HEPES-h buffer (w/v) to make a 40% sucrose/lysate solution. One hundred µl of the sucrose/lysate solution was stored at -20°C as a total membrane input fraction. Sucrose gradients consisted of 3 ml of 5%, 6 ml of 36% sucrose (w/v) in HEPES-h on top of 900 µl of

sample lysate in 40% sucrose 1% Triton X-100 HEPES-h buffer. The gradients were loaded into 17ml Sorvall centrifuge tubes as follows: 3 ml of 5% sucrose was added to the bottom of the centrifuge tube first, 6 ml of the 36% sucrose was pipetted below, and the 1100 µl sample fraction was added last. Centrifuge tubes were loaded onto a Sorvall Surespin 630 rotor and spun at 100 000g for 20 hr at 4°C in a Sorvall Discovery 100S centrifuge. One ml Fractions were collected from the top of the tube with a 'trimmed' P1000 Gilson pipette tip and stored at -20°C for western blot, slot blot, or PCR. The lipid raft fraction was isolated from fraction 3 to 5 that manifested as a flocculent (wispy) band at the 5%-36% sucrose interface.

2.9.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Fractionations were screened for the presence of cell proteins by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. Samples were mixed with 6X loading buffer (0.25 M TRIS-Cl pH 6.8, 50% glycerol, 5% SDS, 0.2% bromophenol blue, 5% β-mercaptoethanol), heated at 90°C for 3-5 min, loaded onto a 3% stacking gel (8% acrylamide (40% acrylamide/bisacrylamide (37.5:1) Biorad), 0.1 M TRIS-Cl pH 6.8, 1.0% SDS, 0.2% TEMED, 0.2% APS) and resolved on a 15% resolving gel (37% acrylamide (40% acrylamide/bisacrylamide (37.5:1) Biorad), 0.4 M TRIS-Cl pH 8.8, 0.1% SDS, 0.1% TEMED, 0.05% APS) beside a Benchmark™ prestained protein ladder (Invitrogen™). Gels were run on a Hoefer Mighty Small II SE 250 (Hoefer Scientific Instruments©, Inc., California, USA) in protein electrophoresis buffer (250 mM TRIS, 2 M glycine, 0.1% SDS) at 25 mA per gel and 70 to 100 V until the sample front reached the bottom of the gel.

2.9.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis of tripartite motif proteins.

Trim proteins were resolved in polyacrylamide gels as above except for the following changes: samples were dissolved directly in 2 X loading buffer, sonicated for 10 min in a Decon F5 minor sonicating water bath (Decon ultrasonics, Hove Sussex, England), and stored at -20°C. When the samples were needed they were boiled for 10 min at 95°C, sonicated for a further 10 min and loaded onto a polyacrylamide gel.

2.9.5 Western blot.

Proteins were transferred to PVDF (poly-vinyl-difluoride) membrane in a Mini Trans-Blot Cell (Biorad Laboratories[®]) as per manufacturers instructions. The (8 x 6 cm) PVDF membrane was activated in methanol then soaked in protein blotting buffer (250 mM TRIS, 2 M glycine, 10% ethanol) for 5-10 min. The running gel was separated from the stacking gel, placed on the activated PVDF membrane, and sandwiched between four pieces of pre-wet 3MM Whatman filter paper (10 x 7.5 cm). This assembly was placed in the blotting cell and blotted for 3 hr at 250 mA and 200 V or overnight at 125 mA and 50 V at 4°C.

The membrane was removed from the blotting assembly and washed briefly in PBS then blocked in PBS, 0.1% TWEEN 20, 5% powdered milk (w/v) for 1 hr with shaking. The membrane was incubated with primary antibody diluted 1/500-1/2000 in PBS, 0.1% TWEEN 20, 5% powdered milk (w/v) for 45 min to 1 hr. The membrane was washed 3 x 10 min in PBS, 0.1% TWEEN 20. The horseradish peroxidase-conjugated secondary antibody was added at a dilution of 1/2000 in PBS, 0.1% TWEEN 20, 5% powdered milk (w/v) for 45 min to 1 hr. The final three washes were done in PBS, 0.1% TWEEN 20 for 10 min each. The last wash was done in PBS only. ECL[™] detection substrate and Hyperfilm ECL[™] (Amersham Biosciences[®]) were used for film detection and ECL-Plus[™] substrate was used for detection of Western blots on a Storm 860[™] (Molecular Dynamics).

2.9.6 Slot blot of fractions derived from sucrose gradient centrifugation.

Dot blotting was used to screen sucrose gradient fractions for lipid raft associated proteins and lipids, as per manufacturers instructions. Briefly, a Bio-Dot SF microfiltration unit (Bio-Rad) was set-up using Bio-Dot SF filter paper and Amersham[®] Hybond-ECL Nitrocellulose membrane as this yielded the best results. The blotting membrane and filter paper were pre-wet in TBS before mounting onto blotting unit. The membrane was washed by drawing 200µl of TBS through each well under suction. An aliquot of each sample was diluted 1/10 in 200µl TBS, drawn through, then washed with 200µl TBS. The membrane was removed from the blotter, washed in TBS for 10 min, and antibody detection was carried out as for a western blot and detection and

densitometry were done on a Storm 860TM (Molecular Dynamics) and ImageQuant software.

2.10 Microscopy

2.10.1 Intracellular antibody staining for major histocompatibility complex class I protein.

HeLa cells were seeded on coverslips. The following day, cells were fixed in freshly prepared solution (3% paraformaldehyde, 60 mM sucrose, 0.1 M sodium phosphate pH 7.2) for 30 min at room temperature. After three washes in PBS for 5 min, cells were washed with PBS 20 mM glycine for 5 min, permeabilised in PBS 20 mM glycine 0.1% saponin for 10 min at room temperature, washed with PBS 20 mM glycine for 5 min and blocked with PBS 20 mM glycine 1% bovine serum albumin (BSA) for 10 min at room temperature. Incubation with primary antibodies was done in blocking solution for 1 h at 37°C. After washing in PBS glycine for 5 min cells were incubated with secondary antibodies (donkey antimouse-FITC) in blocking solution for 45 min at 37°C. Finally, cells were washed for 10 min in PBS and mounted with mowiol (Calbiochem) and analyzed by confocal microscopy (MRC 1024 (BioRad, Hercules, CA, USA) equipped with a krypton-argon laser). Pictures were acquired using Kalman averaging and analysed with Lasersharp software (BioRad). .

2.10.2 Immunofluorescence microscopy.

Cells were visualised after treatment with either FITC-conjugated transferrin (Trf-FITC) or FITC-conjugated cholera toxin B subunit (CTxB-FITC). Slides were mounted with immunofluorescence mounting medium (Dako, Carpinteria, CA, USA) and observed by confocal microscopy [MRC 1024 (BioRad, Hercules, CA, USA) equipped with a krypton-argon laser]. Pictures were acquired using Kalman averaging and analysed with Lasersharp software (BioRad).

2.11 Antibodies

Table 2.7. Antibodies.

Specificity/ (target)	Source/ Ab type	Assay	Dilution	Company	Catalogue no.
Actin	Rabbit pAb	WB	1/2000	Sigma	A-5060
CD4 (Q4120)	Mouse mAb	SB	1/2000	ARP	ARP318
GM1 (CTxB)	CTxB-Biotin (ligand)	SB	1/4000	Sigma	C9972
	CTxB-FITC	IF	1/1000		C1655
HIV-1 (p24 CA)	Mouse mAb	IS	1/100	ARP	EVA 365, 366
HIV-2 antiserum	Human pAb	IS	1/4000	HIV-2 patient	N/A
huMHC-I	Mouse mAb	IF	1/200	Serotec	MCA81
MID 1 (TRIM 18)	Rabbit pAb	WB	1/1000	Orbigen	PAB-11697
MID 2 (TRIM 1)	Goat pAb	WB	1/1000	Imgenex	IMX-3087
PIAP	Mouse mAb	WB/SB	1/2000	Sigma	A2951
RNF 21 (TRIM 34)	Goat pAb	WB	1/1000	abcam	ab4509
Transferrin (TrfR)	Trf-FITC (ligand)	IF	1/1000	Sigma	T0288
HA tag	Mouse mAb	WB	1/3000	Sigma	H9658

pAb = polyclonal antibody; mAb = monoclonal antibody; CTxB = Cholera toxin binding subunit; ARP = AIDS reagent program, NIBSC, Potters Bar, UK; huMHC-I = human MHC-I, N/A = not applicable; PIAP = placental alkaline phosphatase; TrfR = transferrin receptor; WB = western blot; SB = slot blot; IF = immunofluorescence.

2.12 Statistical analysis

The mean and the standard deviation of the mean (SDM) were calculated using Microsoft Excel.

The Spearman's rank coefficient was calculated for HIV-2 V3 loop charge versus MDM tropism as in fig 2.7. The Spearman's rank coefficient tool was used to calculate R_s : http://faculty.vassar.edu/lowry/corr_rank.html.

H_0 : there is no correlation between HIV-2 V3 loop charge and HIV-2 MDM tropism

H_a : there is a negative correlation between HIV-2 V3 loop charge and HIV-2 MDM tropism

X: V3 loop charge	7	6	7	6	8	8	9	8	8
Y: MDM tropism	0.03	0.003	0.008	0.006	0.003	0	0.015	0.0001	0.0004
X: Rank	3.5	1.5	3.5	1.5	6.5	6.5	9	6.5	6.5
Y: Rank	9	4.5	7	6	4.5	1	8	2	3
d	5.5	3	3.5	4.5	2	5.5	1	4.5	3.5
d²	30.25	9	12.25	20.25	4	30.25	1	20.25	12.25

$$(R_s) = 1 - \frac{6 \sum d^2}{n^3 - n}$$

d^2 = the difference between each set of ranks, squared = 139.5

$$R_s = -0.1625$$

Where

$R_s = -1$ to -0.5 = strong negative correlation

$R_s < 0$ to -0.5 = weak negative correlation

$R_s = 0$ = no correlation

Figure 2.7. Spearman rank coefficient for HIV-2 third variable loop charge versus macrophage tropism.

Chapter 3

HIV macrophage tropism and a post-entry restriction to HIV infection in human cells

3.1 Introduction:

HIV-1 HIV-2 both cause AIDS. However HIV-2 causes a slower mean progression to disease and is less transmissible than HIV-1 (Marlink et al., 1994). HIV-2 infection is found primarily in West Africa (Wilkins et al., 1993) (Pfutzner et al., 1992; Piedade et al., 2000). In contrast, HIV-1 infection is found worldwide with particularly high prevalence in sub Saharan Africa. UNAIDS report that at the end of 2004 there were 42 million people living with HIV-1 infection globally (UNAIDS, 2004).

Both viruses use CD4 and a 7-TM coreceptor to gain entry into cells but HIV-2 can use a broader spectrum of coreceptors than HIV-1 *in vitro*, including CCR1, CCR2b, CCR3, CCR5, and CXCR4 (Alkhatib et al., 1996; Clapham and McKnight, 2002; Deng et al., 1996; Maddon et al., 1986; Sattentau et al., 1988). It is likely that CCR5 and CXCR4 are the most important for infection of primary cells but other coreceptors may be used at a lower efficiency (McKnight et al., 1998; Morner et al., 2002).

The HIV-1 and HIV-2 Envs are made up of conserved C1 through to C5 and variable V1 to V5 domains. The HIV-1 third variable loop (V3 loop) acts as a determinant of cell tropism and is the principle neutralising determinant in laboratory isolates (Hwang et al., 1991; Hwang et al., 1992; Javaherian et al., 1990; McKnight et al., 1995; Westervelt et al., 1992). HIV infection normally begins with slow replicating and low titre viruses termed slow/low. A poor prognosis and declining CD4 counts are later associated with the appearance of rapid replicating, high titre viruses termed rapid/high. There is evidence that the HIV-2 V3 loop is an important neutralisation domain as well and an increase in the net positive charge of the V3 loop has been associated with an increase in the rapid/high from a slow/low phenotype, of HIV-2 isolates (Albert et al., 1996; Bjorling et al., 1994)(McKnight, 1995). Isaka et al demonstrated that basic amino acid substitutions in the C-terminal half of the HIV-2 V3 loop would cause a coreceptor

switch from CCR5 to CXCR4 (Isaka et al., 1999). However, it is not known if HIV-2 coreceptor use or V3-loop charge is associated with cell tropism as it is with HIV-1.

HIV-1 infection of MØs has been well documented both *in vitro* and *in vivo* (reviewed by (Martin and Bandres, 1999). The first HIV-1 viruses to emerge in the host are MØ tropic (M-tropic), CCR5 using, probably because during transmission, CCR5 expressing MØs and DCs, resident in the mucosae, are the first likely targets. Although there are some exceptions, HIV-1 MØ tropism results from the ability to use CCR5 as a coreceptor. Like mucosal transmission, MØ tropic CCR5 using viruses isolates emerge in preference to CXCR4 using T cell tropic isolates, during intravenous transmission of HIV-1. In this case it is speculated that both T cell tropic and MØ tropic isolates are transmitted but the isolates that infect MØs flourish. It has been proposed that the transmitted MØ tropic virus pool facilitates CTL escape (Schutten et al., 2001), ((Schuitemaker, 1994). It has been demonstrated that HIV-2 can infect both MDMs and T cells *in vitro* but it is not known what role MØs play in HIV-2 infection (Castro et al., 1990; Valentin et al., 1994) and the relevance this has to HIV-2 transmission is unknown.

In about 50% of untreated HIV-1 subtype B infections there is a switch from MØ tropic, CCR5 using viruses to T cell tropic, CXCR4 using viruses. The change in cell tropism and coreceptor usage of HIV-1 is often accompanied by a poor prognosis and progression to AIDS (Nielsen et al., 1993). The picture for HIV-2 is less well explained but one study shows that HIV-2 isolates taken from AIDS patients use CXCR4 whereas isolates from asymptomatic patients use CCR5 and CCR3 (Blaak et al., 2005; Sol et al., 1997). Thus HIV-2 may follow the same coreceptor switch during infection. Whether the switch from CCR5 to CXCR4 usage in HIV-2 infection is associated with a switch from M-tropism to T-tropism is not known.

The elevation in HIV-1 or HIV-2 RNA plasma viral load is associated with the decline in the CD4 count that is the hallmark of the onset of illness (Schuitemaker et al., 1992) (Damond et al., 2002). It appears that the CD4 count of patients with HIV-2 remains higher than the CD4 count of patients with HIV-1 regardless of recent sero-conversion or development of AIDS (Andersson et al., 2000).

All of the Lentiviruses have a propensity to replicate in MØs and indeed there are Lentiviruses that mediate their infection and pathogenicity solely through these cells. The non-primate Lentiviruses Maedi-Visna Virus, Caprine-Arthritis Encephalitis Virus, and Equine Infectious Anaemia Virus (EIAV) all mediate their pathogenesis by infecting MØs. HIV uses MØs as a key infectable cell type and so the propensity of HIV-1 and HIV-2 to infect MØs may be a pathogenesis determinant.

In this chapter I characterise the replication of HIV-2 primary isolates in MØs and T cells from PBMC and compare it with HIV-1. Thus by comparing the MØ tropism of HIV-1 with the less pathogenic HIV-2 this study may provide insight in to why HIV-1 is significantly more pathogenic than HIV-2. MDM infected with HIV-2 primary isolates produced an early and brief burst of replication, followed by a latency, without rebound at later time points whereas HIV-1 primary isolates showed a delayed burst of replication in MDM but showed stable virus production over 21 days. The V3 loop amino acid charges of the HIV-2 isolates were calculated from the sequences and there appeared to be an association with coreceptor usage and a weak negative correlation with MØ tropism, similar to HIV-1.

3.2 Results:

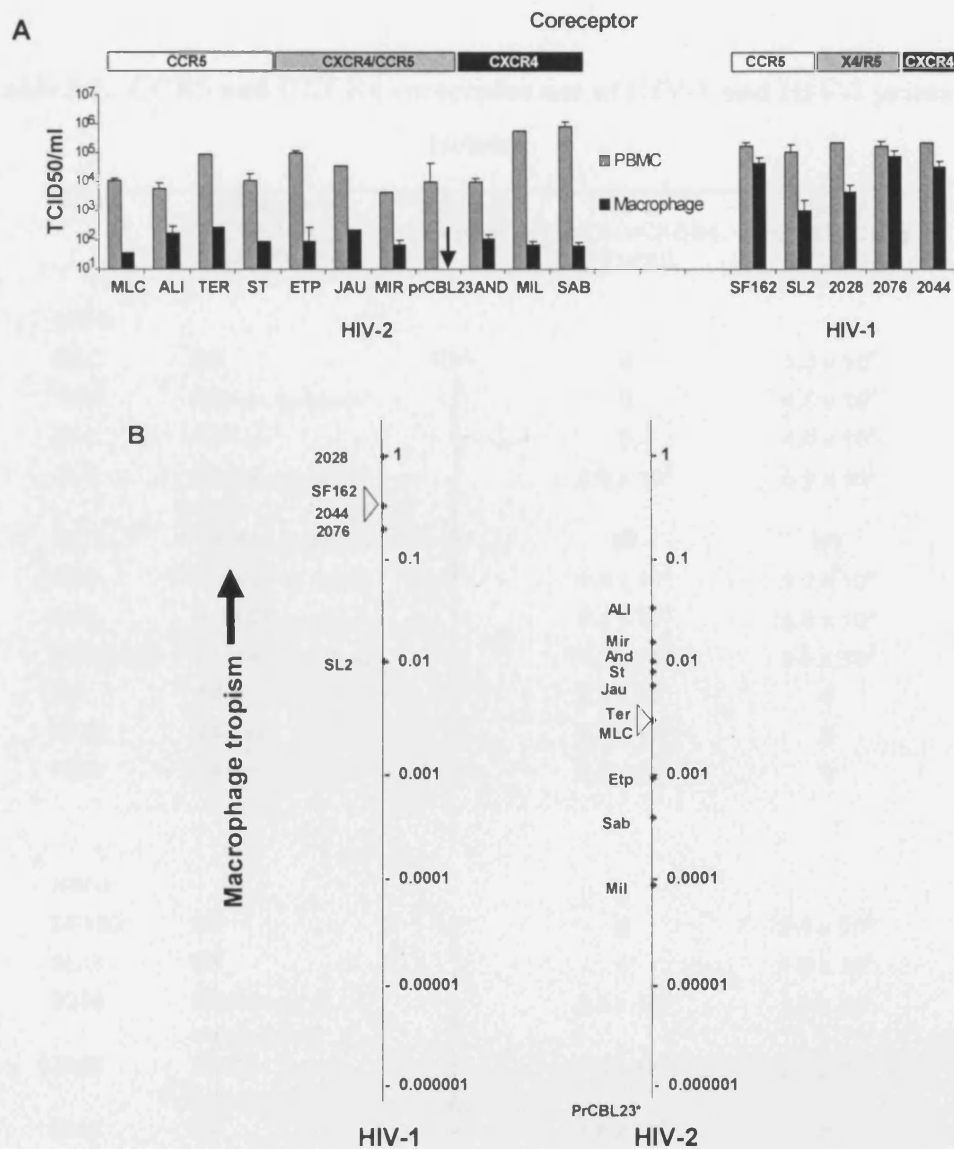
3.2.1 HIV-2 primary isolates, compared to HIV-1, exhibit poor replication in monocyte-derived macrophages.

Infection of MDMs by both R5 and X4 HIV-1 primary isolates has been previously described (Clapham PR, 2001; Eckstein et al., 2001; Valentin et al., 1994; von Briesen et al., 1990). First I determined how well HIV-2 primary isolates infect MDMs. I directly compared the infection of MDM to PBMC by HIV-1 and HIV-2. The viruses used here represent a broad range (table 3.1); a spectrum of HIV-2 primary isolates was selected that use CCR5, CXCR4, or both coreceptors. The HIV-1 primary isolates chosen also have a broad spectrum of coreceptor use and MØ tropism (McKnight et al., 1998), (Reeves et al., 1999) and (Simmons G, 1998). Supernatants were harvested and RT activity was determined by RT-ELISA (Caviditech). TCID₅₀ for each virus was

determined on both MDMs and PBMC at 21 and 14 days post-infection, respectively, as has been described for HIV-1 (fig 3.1a) (Simmons G, 1996).

The HIV-1 isolates efficiently infected MDM whereas the HIV-2 isolates did not, even though HIV-2 had higher titres on PBMC. HIV-2 MIL and SAB had TCID₅₀s on PBMC of 5.6×10^5 and 8.6×10^5 respectively, compared to the HIV-1s 2028 and 2076 that had TCID₅₀s on PBMC of 2.2×10^5 . HIV-2 was approximately 1 to 2 logs less infectious for MDMs than any HIV-1 isolate (fig 3.1a). HIV-2 TER shows the highest HIV-2 TCID₅₀ on MDM of 2.8×10^2 and the lowest TCID₅₀ is that of HIV-2 MLC, 3.6×10^1 . PrCBL 23 does not have a TCID₅₀ that can be calculated from MDM infection since no infection could be detected. This is in contrast to HIV-1 where the lowest TCID₅₀ on MDM is SL2, 1.0×10^3 , the highest is 7.4×10^4 of 2076.

To better compare HIV-2 MDM infection the ratio of MØ to PBMC tropism was determined from the values in fig 3.1a. The value of the MDM TCID₅₀ was divided by the PBMC TCID₅₀ for each isolate and the relative MØ tropism values were plotted (fig 3.1b). The most 'macrophage tropic' HIV-2 was Ali, and then in order of descending MØ tropism were MIR, AND, ST, JAU, TER, ETP, MLC, SAB, and MIL. PrCBL 23 could not infect MDM even when titres of 10^4 TCID₅₀ were reached on PBMC.



*PrCBL23 does not register on a logarithmic chart since its relative macrophage tropism value is 0.

Figure 3.1. HIV-2 is less efficient at infection of monocyte derived macrophages than HIV-1.

(A) Tissue culture infectious dose 50s per ml (TCID₅₀/ml) of HIV-2 and HIV-1 primary isolates on MDM and PBMC, harvested at 21 days (MDM) and 14 days (PBMC) post-infection. Coreceptor use is indicated (see table 3.1). HIV replication was detected by RT ELISA. (B) Tropism scatter plot. To illustrate the difference between HIV-2 and HIV-1 MØ tropism a scatter plot was made from the PBMC and MDM TCID₅₀s from fig 3.1a. This plot was constructed by dividing the MDM TCID₅₀ by the PBMC TCID₅₀. As previously described (Simmons G, 1996).

Table 3.1. CCR5 and CXCR4 coreceptor use of HIV-1 and HIV-2 primary isolates.

Isolate	Coreceptor(s) CCR5 (R5), CXCR4 (X4)	Preferred coreceptor	Titre on NP2/CD4/CXCR4 (FFU/ml)	Titre on NP2/CD4/CCR5 (FFU/ml)
HIV-2				
MLC	R5	R5* ↓	0	1.0 x 10 ⁴
TER	R5+ R1, R3, CXCR6		0	4.5 x 10 ⁴
ALI	R5+ R1		0	4.0 x 10 ⁴
JAU	R5/X4+ R1, R2b, R3, R8		5.0 x 10 ³	5.0 x 10 ³
ST	R5/X4+ R1, R2b, R3		NT	NT
ETP	R5/X4+ R1, R2b, R3		1.0 x 10 ⁵	5.0 x 10 ²
MIR	R5/X4+ R1, R2b, R3		5.5 x 10 ⁴	5.0 x 10 ²
PrCBL-23	R5/X4+ R1, R2b, R3		4.0 x 10 ³	5.0 x 10 ²
MIL	X4		2.3 x 10 ⁴	0
SAB	X4+ R3	↓ X4	4.0 x 10 ⁴	0
AND	X4+ R1, R2b, R3		1.0 x 10 ⁵	0
HIV-1				
SF162	R5	R5* ↓	0	2.5 x 10 ⁵
SL-2	R5		0	1.0 x 10 ⁵
2076	R5/X4+ R3, R8, STRL-33, GPR-15		2.0 x 10 ⁴	1.8 x 10 ⁴
2028	R5/X4+ R3, R8, STRL-33, GPR-15		7.5 x 10 ⁴	7.3 x 10 ⁴
2044	X4		7.8 x 10 ⁴	0

'+' denotes additional coreceptor use as determined previously (Reeves et al, 1999; McKnight et al, 1998; Neil et al, 2005), NT=not tested, Coreceptor shown in bold denotes predominant use of that coreceptor.

3.2.2 Like HIV-1, the third variable loop charge on the HIV-2 envelope may be associated with HIV-2 macrophage tropism.

I set out to determine if the V3 loop charge of HIV-2 is associated with MØ tropism because it has been shown that HIV-1 tropism, coreceptor usage, and V3 loop charge are associated (Bieniasz et al., 1997; Kuiken et al., 1992; Shioda et al., 1992). A high V3 loop charge on all of the HIV-2 isolates used in this study it may explain the lack of

MØ tropism of HIV-2. DNA was harvested from HIV-2 infected PBMC 48 hr post-infection and the V3 loop region was sequenced from the HIV-2 proviral DNA. The number of negatively charged amino acids were subtracted from the positively charged amino acids, between the V3 loop cysteines, to obtain the net V3 loop charge. Table 3.2 illustrates how the coreceptor usage of the HIV-2 primary isolates studied is associated with the amino acid charge, ranging from +6 to +10, on the Env V3 loop. The charge increase is seen primarily in the C-terminal half of the loop and is associated with increased CXCR4 use by HIV-2. ETP was the only HIV-2 isolate that exhibited a negatively charged amino acid. There is no consistently high V3 loop charge pattern but a spectrum of charges.

Table 3.2. HIV third variable loop sequences, charge and coreceptor usage.

HIV-2	Coreceptor*	V3 Sequence [†]	V3 Charge
ALI	R5 +	CKRPGNKTVPITLMSG LI FHSQ P INK-RERQAWC	+7
MLC	R5	CKRPGNKTVPITLMSG LV FHSQ P INN-RERQAWC	+6
ST	R5/(X4) +	CKRPGNKTVPITLMSG LV FHSQ P INR-RERQAWC	+7
JAU	R5/X4 +	CKRPGNKT VL PITLMSG LV FHSQ P PLNT-RERQAWC	+6
ETP	(R5)/X4 +	CKRPGNKA V KPITLMSG FK FHSR P VINIKERQAWC	+8
PrCBL20	X4/(R5) +	CKRPGNKT VL PITLGS GR FHAQ P IIIS-KERQAWC	+8
PrCBL23	X4/(R5) +	CKRPGNKTVPITLMSG LR FHSQ P VINKRERQAWC	+8
MIR	X4/(R5) +	CKRPGNKT VL PITLMSG RK FHSQ P IINKRERQAWC	+9
MIL	X4	CKRPGNKTVPITLMSG AK FHSR P VINTK E KQAWC	+8
SAB	X4 +	CKRPGNKTVPITLMSG AK FHSR P VINN K EKQAWC	+8
A-nd	X4 +	CKRPGNKS V LPITLMSG HR FHGCKIINKRERQAWC	+10

HIV-1	Coreceptor	V3 Sequence [†]	V3 Charge
SF162	R5	CTRPNNNTRK S ITIGPGR A FYATG D IIG D IRQAH C	+4
GUN-1wt	R5/X4	CTRPNNNTRK S ITIGSGR A FHA I KIIGNIRQAH C	+7
SF2	X4	CTRPNNNTR S ISTGPGK A LHITGRIIG D ITKAH C	+5
IIIB	X4	CTRPNNNTRK S IRIQRGPR A FVTIGKIGNIRQAH C	+9

Ψ ~~Positively charged amino acid~~
Negatively charged amino acid

* (R5), (X4) denote at least 10 fold less efficient CCR5 or CXCR4 usage than main coreceptor (table 3.1).

† Sequences from National Centre for Biological Information (NCBI), NIH. Accession no: SF162 AY988107, GUN-1wt D34598, IIIB M64769, SF2 K02007

Interestingly, when the coreceptor use of HIV-2 in table 3.1 is compared to the MØ tropism in fig 3.1b there is a general correlation between CCR5 use and MØ tropism (table 3.2), those that use CCR5 tend to be the most MØ tropic as indicated by a slight

downward slope of the regression trend line in fig 3.2. However there are some anomalies: MLC uses CCR5 preferentially to CXCR4 but has rated low on the MØ tropism scale and MIR, a CXCR4 using isolate with the highest V3 loop charge is the second most MØ tropic HIV-2. Although, all of the HIV-2 isolates tested (except prCBL 23) demonstrated some replication in MDM none could replicate to levels comparable to HIV-1.

To determine whether HIV-2 V3 loop charge is negatively correlated with MØ tropism I used the Spearman's rank correlation (materials and methods). H_0 states that there is no correlation between HIV-2 V3 loop charge and MØ tropism, and H_a states that there is a negative correlation between the two. The Spearman's rank correlation returns a coefficient (R_s) of -0.1625 . Where $R_s=0$ there is no correlation, and R_s between -0.5 and 0 is a weak negative, and R_s between -0.5 and -1.0 is a strong negative correlation. Therefore I reject the null hypothesis and confirm that there is a weak negative correlation between HIV-2 V3 loop charge and MØ tropism (fig 3.2). When the V3 loop charges of two HIV-1 isolates SF2 and SF162 were compared to their MØ tropism the Spearman's rank coefficient returned a value of -0.67 , which confirms a strong negative correlation between HIV-1 V3 loop charge and MØ tropism, as described previously (Hwang et al., 1991; Hwang et al., 1992; Westervelt et al., 1992).

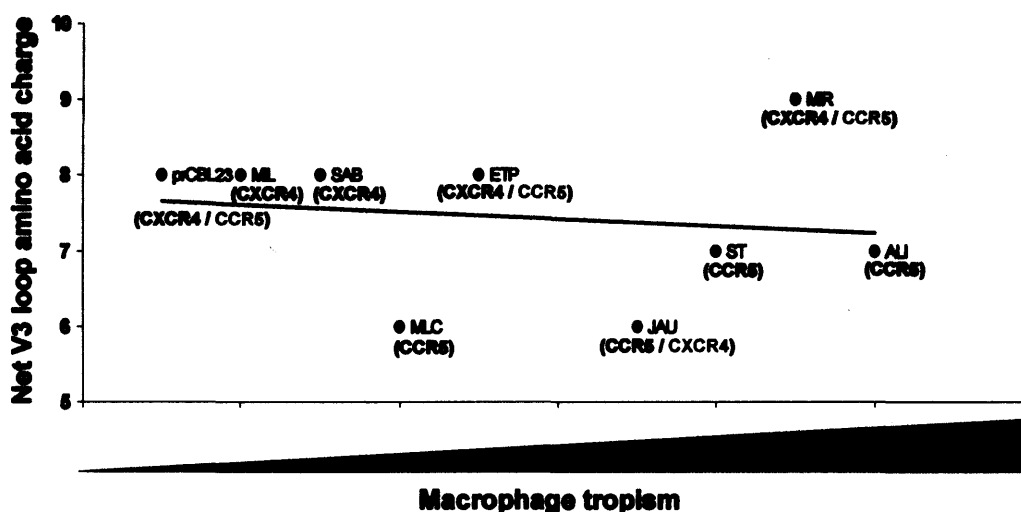


Figure 3.2. Macrophage tropism versus the third variable loop charge of HIV-2.

The MØ tropism from fig 3.1b was plotted against the net V3 loop amino acid charge in table 3.2. The net V3 loop amino acid charge was calculated by subtracting the total charge of the negatively charged amino acids from the total of the positively charged amino acids. The coreceptor usage, listed beside

each isolate, was taken from table 3.1. The coreceptor used with greatest efficiency is listed first in bold black type. Additional coreceptors are listed in grey. The regression trend line was plotted with Microsoft Excel. A weak negative correlation between HIV-2 V3 loop charge and MØ tropism is supported by a Spearman's rank coefficient of -0.1625 (above).

3.2.3 HIV-2 reverse transcription in monocyte derived macrophages is transient.

Limiting dilution *gag*-LTR PCR was used to investigate the efficiency of the HIV-2s MIR, TER, MIL, and ETP to enter and reverse transcribe in MDM compared with PBMC and with HIV-1. The ERV-3 PCR was used as a DNA input control and to show that the host genomic DNA input is equivalent between time points, cell types and isolates. ERV-3 is present in one copy per haploid human genome (chromosome 7) that allows for comparison to the number of HIV genomes present (Cohen et al., 1985; O'Connell et al., 1984). Fig 3.3 shows that a similarly high level of HIV-2 MIR *gag*-LTR DNA was produced at 24hrs post-infection in both MDM and PBMC. At 72 hrs MIR *gag*-LTR continued to increase in PBMC but there was a decrease of approximately 2.0 logarithms of HIV-2 *gag*-LTR DNA in MDM. This same pattern of reverse transcription in MDM was seen with all HIV-2s tested. Thus the HIV-2s enter and reverse-transcribe in MDM regardless of the V3 loop charge and coreceptor usage.

PBMC and MDM infected with HIV-1 2044 were subject to limiting dilution PCR as with HIV-2 above to compare 24 hr and 72 hr time points. I wanted to determine if the *gag*-LTR product was lost in HIV-1 infected MDM similar to HIV-2 infected MDM. In contrast, the amount of *gag*-LTR product increases in PBMC at 72 hr compared to 24 hr (fig 3.3). There was approximately a 3-fold loss of *gag*-LTR product in MDM at 72 hr compared to 24 hr, however there was approximately 3-fold less input DNA (see ERV-3 control) at this time point suggesting no relative loss in HIV-1 *gag*-LTR product. Therefore, there was less loss of reverse transcription product with HIV-1 infection of MDM compared to HIV-2.

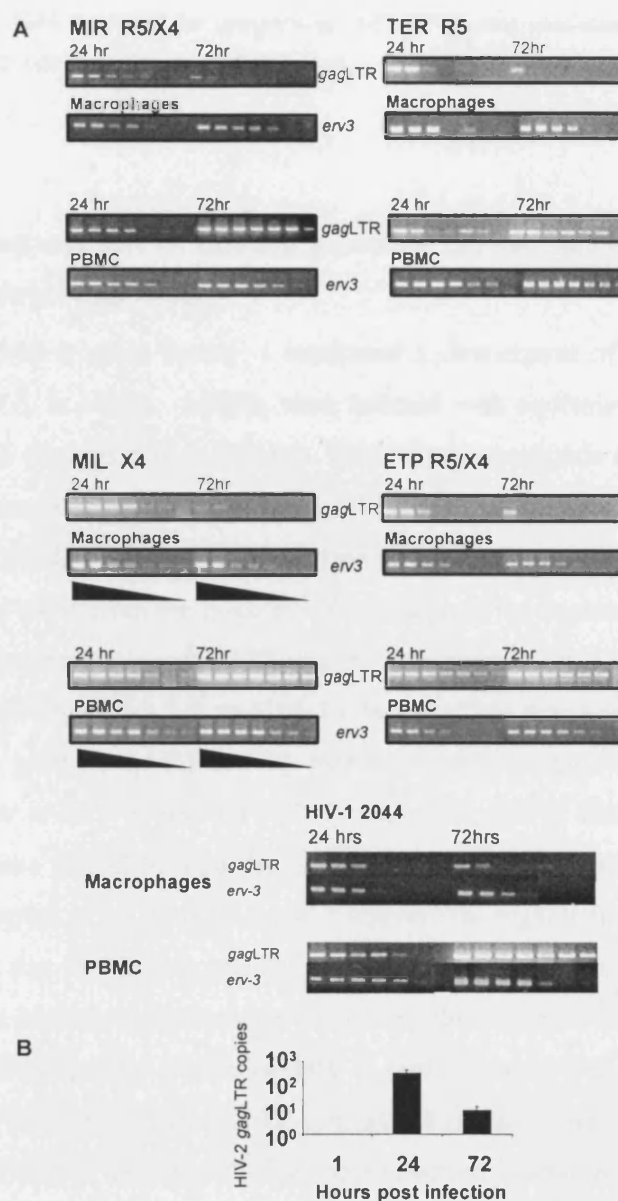


Figure 3.3. Limiting dilution *gag*-LTR polymerase chain reaction and ERV-3 polymerase chain reaction analysis of HIV-2 time courses on monocyte derived macrophages and peripheral blood mononuclear cells.

(A) To compare the extent of HIV-2 reverse transcription in PBMCs and MDM I measured the final stages of reverse transcription by *gag*-LTR PCR. PBMCs and MDM (10^6 cells each) were infected with 10^3 TCID₅₀ of HIV-2 virus [HIV-2 titres were determined in PBMC and coreceptor usage was determined in NP2/CD4/CXCR4 and NP2/CD4/CCR5 cells (fig 3.1a and table 3.1)]. Cells were infected with HIV-2 inoculum for 3 hr and then washed twice with RPMI/10% HS. Serial dilutions were made of the infected PBMC or MDM DNA in half logarithm dilutions starting with neat and ending with a dilution of 10^{-3} .

MØ tropic HIV-1 2044 is shown for comparison. (B) QPCR was conducted on DNA from MDM infected with HIV-2 MIR above, to verify serial dilution method in fig 3.3a.

3.2.4 HIV-2, but not HIV-1, infected monocyte derived macrophages exhibit an early burst in viral replication.

To investigate MØ tropism further, I conducted a time-course of virus replication on HIV-2 and HIV-1 in MDM. MDMs were infected with equivalent amounts of either HIV-1 or HIV-2 (determined on PBMC). The input corresponds to approximately 10^4 TCID₅₀/ml as measured on PBMC for each HIV-2 isolate. The inputs for MIL and SAB were 5.0×10^4 and 8.0×10^4 TCID₅₀/ml respectively. At each time point, an aliquot of supernatant was taken from the cultures. RT activity of the supernatants was measured by RT-ELISA and a time course of RT activity was plotted. Fig 3.4a shows that there is a burst of RT activity at day 2 from HIV-2 infected MDM that was not detected at later time points. In general, the RT activity measured at the burst of replication for HIV-2 was higher than in HIV-1 infected MDM. However, HIV-1 MDM virus production rebounded at time points beyond the initial replication peak (fig 3.4b). HIV-2 ALI produced 150 pg/ml of RT activity at day 2 whereas the highest for HIV-1 is 2076 with 50 pg/ml RT at day 16 (fig 3.4a and b). As seen here, the infectivity of HIV-2 primary isolates may not be truly represented by calculating the TCID₅₀ at 21 days after infection but by conducting a time-course of HIV-2 replication in MDM (fig 3.4a). The continuous and late replication of HIV-1 in MDM has been well established thus the measurement of HIV-1 TCID₅₀s 21 days post-infection is appropriate here (Gorry PR, 2001; Simmons G, 1996).

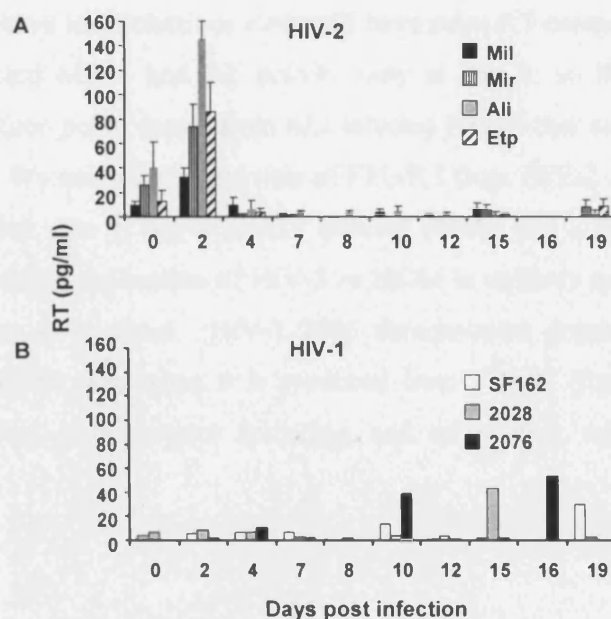


Figure 3.4. HIV-2 replication in monocyte derived macrophages is early and transient.

(A) Time courses of HIV-2 particle RT production and (B) HIV-1 RT production in MDM. Aliquots were taken at 2 day intervals for RT ELISA. Time 0 was measured after inoculum was left on MDM for 3 hr followed by two washes. (C) Particle infectivity. The ratio of infectivity (FFU/ml) to virus particles [RT activity (pg/ml)] of output HIV-2 ALI from PBMC and MDM was used to determine that HIV-2 virus production in MDM resulted in infectious particles. One time point from ALI in PBMC (day 4) is compared to a time point from ALI in MDM (day 2), and HIV-1 2076 (day 16). Shown is the result of one experiment on 2 different MDM and PBMC donors. This result is representative of the results from at least 4 separate experiments using 2 MDM/PBMC donors per experiment. Error bars represent SDM.

3.2.5 The lack of HIV-2 spread in monocyte derived macrophage culture is not because of defective assembly.

It was possible that HIV-2 virion assembly in MDM was inefficient and thus HIV-2 could not spread through MDM culture by repeated rounds of replication in surrounding cells. To determine if infectious virus was produced by HIV-2 ALI infected MDM, harvested supernatants were plated onto NP2/CD4/CCR5 cells and the FFU/ml estimated. RT ELISA was used to quantify RT activity. The RT activity was compared to the number of infectious particles (FFU) by dividing the FFU at a time point by the RT activity (pg/ml) from the same time point (infectivity per unit of RT, i.e. virus

supernatants that have less infectious virus will have more RT compared to infectivity). HIV-2 ALI infected MDM had RT activity only at day 2, so this time point was compared to one time point, day 4, from ALI infected PBMC that exhibited rebounding virus replication. We can see that the ratio of FFU/RT from HIV-2 ALI infected MDM is equivalent to that seen at day 4 in ALI infected PBMC (fig 3.5b), thus the lack of spread and rebounding replication of HIV-2 in MDM is unlikely to be the inability to produce infectious virus alone. HIV-1 2076 demonstrates greater infectivity when produced from MDM than when it is produced from PBMC (fig 3.5b), which may explain why HIV-1 demonstrates spreading and rebounding replication in MDM culture.

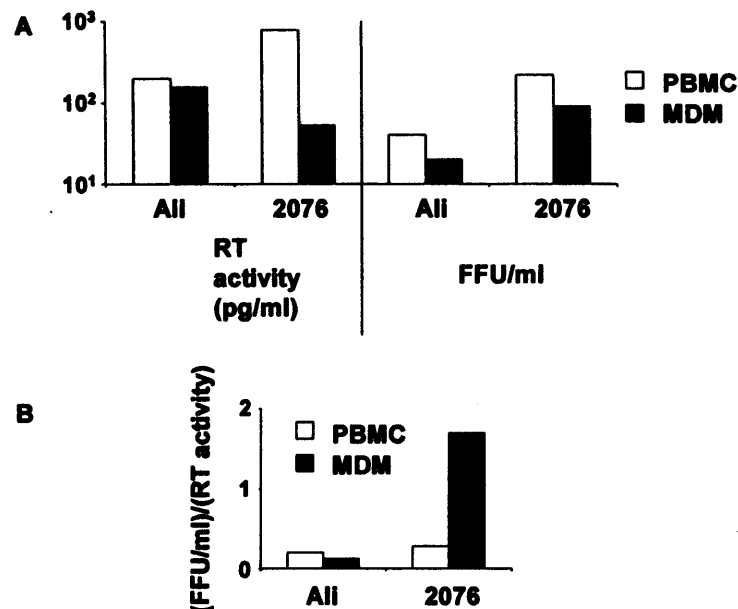


Figure 3.5. HIV-2 ALI produces infectious particles from infected monocyte derived macrophages.

The ratio of infectivity (FFU/ml) to RT activity (pg/ml) of HIV-2 ALI harvested from infected PBMC and MDM was used to determine that HIV-2 virus production in MDM resulted in infectious particles comparable to PBMC (bottom panel). One time point from ALI infected PBMC (day 4) is compared to a time point from ALI infected MDM (day 2), and HIV-1 2076 (day 16) (top panel). Shown are the results from samples obtained from the experiment in fig 3.4 and are representative of the result from 2 independent experiments using 2 MDM and PBMC donors each.

3.2.6 Latent virus production can be stimulated in HIV-2 infected monocyte derived macrophages.

To determine whether latent integrated HIV-2 provirus was present in MDM culture, lipopolysaccharide (LPS) was added to a well of infected MDM 4 days post-infection and harvested the following day (day 5 post-infection). Stimulation of transcription of HIV-2 JAU RNA was evident by *gag*-LTR RT-PCR (fig 3.6a). The MØ tropic HIV-1 SF162, maintains *gag*-LTR RNA production throughout the time-course, indicating constant viral replication (fig 3.6b).

3.2.7 There is a loss of HIV-2 *gag*-LTR RNA production with HIV-2 *gag*-LTR DNA loss.

I next determined whether viral RNA transcripts were being produced over a 5 day period because, as fig 3.4a indicates, there is a burst of RT production at early time points but not at late ones. I wanted to determine if HIV-2 JAU RNA transcription was associated with RT production. RT-PCR to measure *gag*-LTR RNA transcripts was conducted on MDM infected with HIV-2 JAU or HIV-1 SF162. MDM were incubated with equivalent input (5.0×10^3 PBMC TCID₅₀) of either JAU or SF162 for 3 hr, time points were harvested every 24 hr up to and including 5 days post-infection. The infected MDM were harvested with Trizol[®] RNA extraction reagent and the cDNA was prepared. HIV-2 RNA production by MDM peaks at 24 hr post-infection and decreases to almost undetectable levels by day 5 (fig 3.6a). Interestingly, JAU transcription could be restimulated by addition of LPS and titrated out 100-fold (fig 3.6a), however the amount of recovery is not as intense as the expression seen at 24 hr post-infection.

I determined whether the LPS stimulation of HIV-2 viral RNA production in fig 3.5a translated to production of virus particles. I detected virus particles in harvested supernatants by RT-ELISA and show that HIV-2 JAU can be stimulated to higher levels than HIV-1 SF162 at day 5 with LPS stimulation (fig 3.6c). SF162 was apparently unaffected by LPS treatment.

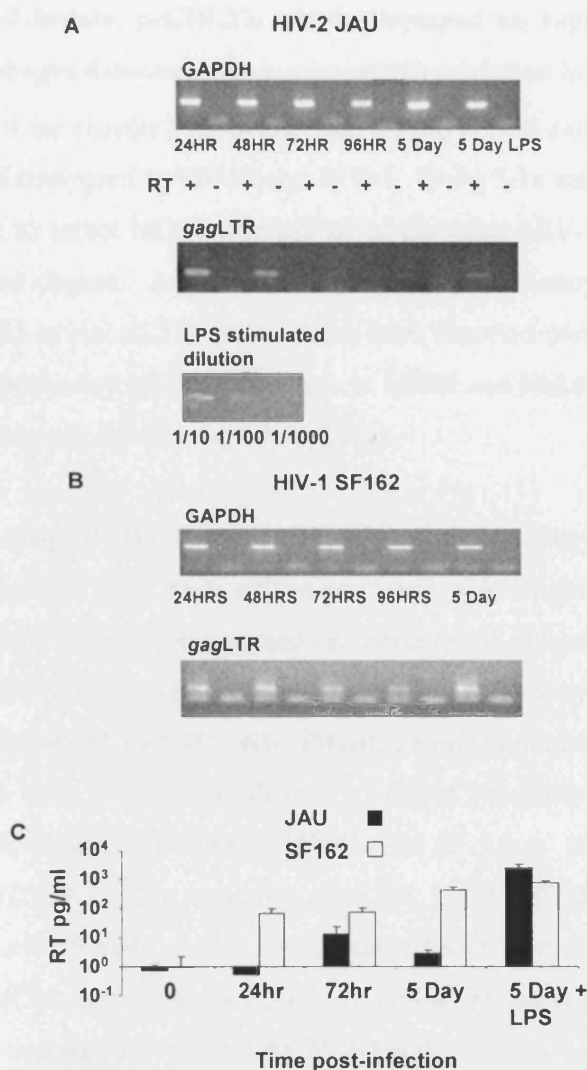


Figure 3.6. HIV-2 infection of monocyte derived macrophages is transient and can be restimulated with lipopolysaccharide treatment.

RT-PCR of (A) HIV-2 JAU and (B) HIV-1 SF162 infected MDM harvested at indicated time points post-infection. LPS (50ng/ml) was added to the HIV-2 JAU culture 4 days post-infection and harvested on day 5. RNA was extracted at the time points indicated and cDNA was made for GAPDH and *gag*-LTR PCR. (C) RT activity from the experiment in fig 3.6a and b above. Shown are the results of one experiment and they are representative of the results from at least 3 independent experiments. Error bars represent SDM.

3.2.8 The HIV-2 isolate, prCBL23, which displayed no replication on monocyte derived macrophages demonstrates a post-entry restriction in HeLa/CD4 cells.

Until this point of the chapter I have described a general post-entry restriction of HIV-2 isolates in MDM compared to PBMC and HIV-1. In fig 3.1a and b I noted that prCBL 23 was not able to infect MDM whereas all of the other HIV-2s tested were able to replicate, to some degree. At this point I focus on a post-entry restriction of HIV-2 prCBL 23 (MCR) in HeLa/CD4 cells. It has been reported previously that prCBL 23 demonstrates a post-entry block to infection in MDM and HeLa/CD4 cells that occurs post-reverse transcription (McKnight et al, 2001).

The T cell line adapted HIV-2 strain CBL 23 and the primary isolate prCBL 23 are restricted in HeLa/CD4 cells, as described previously (McKnight et al, 2001). To study this post-entry restriction I concentrated on infection of HeLa/CD4 cells as they are relatively easy to culture. Fig 3.7 shows that CBL 23 has very similar infectivity for U87/CD4/CXCR4 and HeLa/CD4 cells. PrCBL 23 infection of U87/CD4/CXCR4 cells is comparable to CBL 23, however there is a drop in infectivity from a mean of 2.3×10^4 FFU on permissive U87/CD4/CXCR4 cells to 5.0×10^2 FFU on restrictive HeLa/CD4 cells (fig 3.7). The mean titre of prCBL 23 on U87/CD4/CXCR4 divided by the titre on HeLa/CD4 cells reveals a restriction of 45.6-fold. Thus, the restriction of prCBL 23 in MDM compared to PBMC is similar to the restriction, seen here, in HeLa/CD4 cells compared to U87/CD4/CXCR4 cells

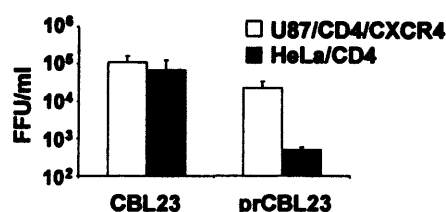


Figure 3.7. HIV-2 PrCBL 23 is restricted in HeLa/CD4 cells.

CBL 23 and prCBL 23 viruses were titrated onto U87/CD4/CXCR4 and HeLa/CD4 cells, fixed and stained for infection 3 days later. Foci of infection were counted and FFU/ml calculated.

3.2.9 Lv2 is a restriction to HIV infection in some human cell types.

Molecular clones and gene swaps were made from prCBL 23 and CBL 23 by my colleague, Christian Schmitz, to study the restriction of prCBL 23 in MDM and HeLa/CD4 cells. These molecular clones were named Molecular Clone Restricted (MCR) and Molecular Clone Not-restricted (MCN) after prCBL 23 and CBL 23, respectively (Schmitz et al., 2004). Restrictive cells are resistant to infection by restricted viruses, MCR and prCBL 23 on HeLa/CD4 cells. This pattern of HIV restriction in human cells has been named Lentivirus susceptibility factor 2 (Lv2) (Schmitz et al., 2004), after the Lentivirus susceptibility factor 1 (Lv1) pattern of retroviral restriction in non-human primate cells (Cowan et al., 2002). Figs 3.8a and b show infection of a standardised dose (equivalent input determined on unrestrictive U87/CD4/CXCR4) of MCR and MCN on the permissive U87/CD4/CXCR4 and the Lv2 restricted HeLa/CD4 cells. As expected MCN is not restricted on either of the U87/CD4/CXCR4 or HeLa/CD4 cells but there is 42.0-fold less infection of MCR on the HeLa/CD4 than the U87/CD4/CXCR4 cells.

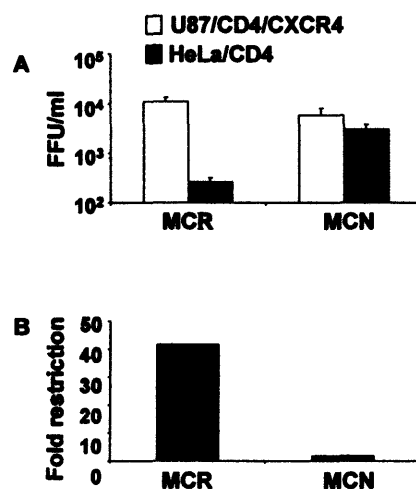


Figure 3.8. Lv2 is a restriction to HIV infection in some human cell types.

(A) The same infectious dose (standardised on permissive U87/CD4/CXCR4 cells) of MCR and MCN were plated on permissive U87/CD4/CXCR4 and restrictive HeLa/CD4 cells and fixed and stained for HIV-2 infection 3 days later. Infectivity is reported as FFU/ml. (B) The fold restriction was calculated by dividing the FFU/ml on permissive U87/CD4/CXCR4 cells by the FFU/ml on restrictive HeLa/CD4 cells for each virus in (A). Results shown represent at least 10 independent experiments. Error bars represent SDM.

3.2.10 The viral *gag* and *envelope* genes act in concert to mediate Lv2 restriction.

All of the Lv2 restriction is accounted for by swapping both the *env* and *gag* genes into the unrestricted MCN backbone (MCNmcrenv*gag*) (Schmitz et al., 2004). Figs 3.9a and c show that the MCNmcr*gag* and MCNmcrenv swaps are restricted by only 17.0 and 11.0-fold, respectively. However, the double *gag* and *env* swap in figs 3.9b and c (MCNmcrenv*gag*) is restricted by 34.0-fold, similar to MCR. Conversely, the MCRmcrenv*gag* swap is not restricted, like the unrestricted MCN in fig 3.8 (figs 3.9b and c).

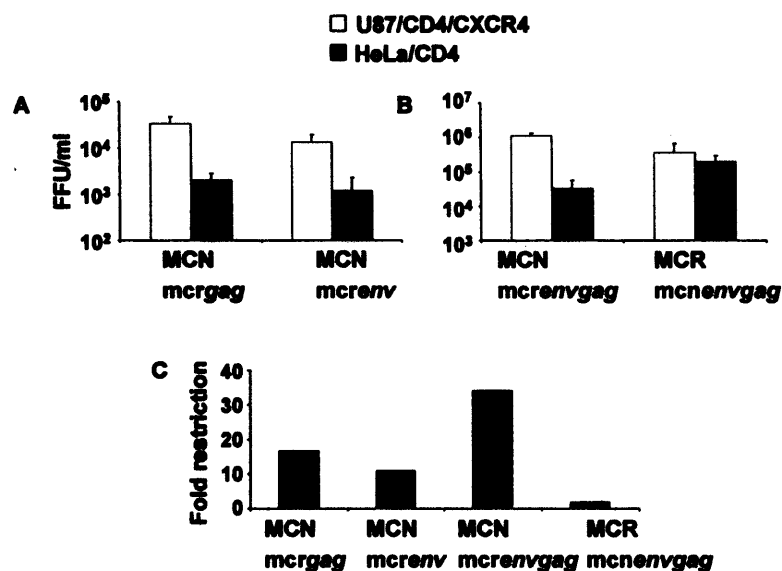


Figure 3.9. HIV-2 *gag* and *envelope* act in concert to mediate the restriction of MCR in HeLa/CD4.

The same infectious dose (standardised on permissive U87/CD4/CXCR4 cells) of (A) *gag* and *env* gene swaps MCNmcr*gag* and MCNmcrenv, and (B) MCNmcrenv*gag* and MCRmcrenv*gag* were plated on U87/CD4/CXCR4 and restrictive HeLa/CD4 cells, fixed and stained for HIV-2 infection 3 days later. Infectivity is reported as FFU/ml. (C) The FFU on permissive U87/CD4/CXCR4 cells was divided by the FFU obtained from restrictive HeLa/CD4 cells for each virus in (A, B) and plotted as fold restriction. Results shown represent at least 5 independent experiments. Error bars represent SDM.

3.2.11 Lv2 restriction of HIV infection is common among HIV-1 and HIV-2 isolates.

HIV-1 (NL4.3, RF, IIIB, MN, SF2, 2044, 2005, 2028, 2076, M13, HAN2, and 89.6) and HIV-2 (MCR, MCN, MIL, ETP, JAU, ACR23, MIR, AND, prCBL20 and SAB) laboratory adapted and primary isolates were tested for infection on U87/CD4/CXCR4, HeLa/CD4 and H399 cells to determine the prevalence of Lv2 restriction (fig 3.10a). The fold restriction of infection on HeLa/CD4 and H399 cells was determined and half of all of the HIV-2 isolates (MCR, ETP, AND, prCBL20, and SAB) and about one third of the HIV-1 isolates (2076, M13, HAN2, and 89.6) were restricted in an Lv2 manner.

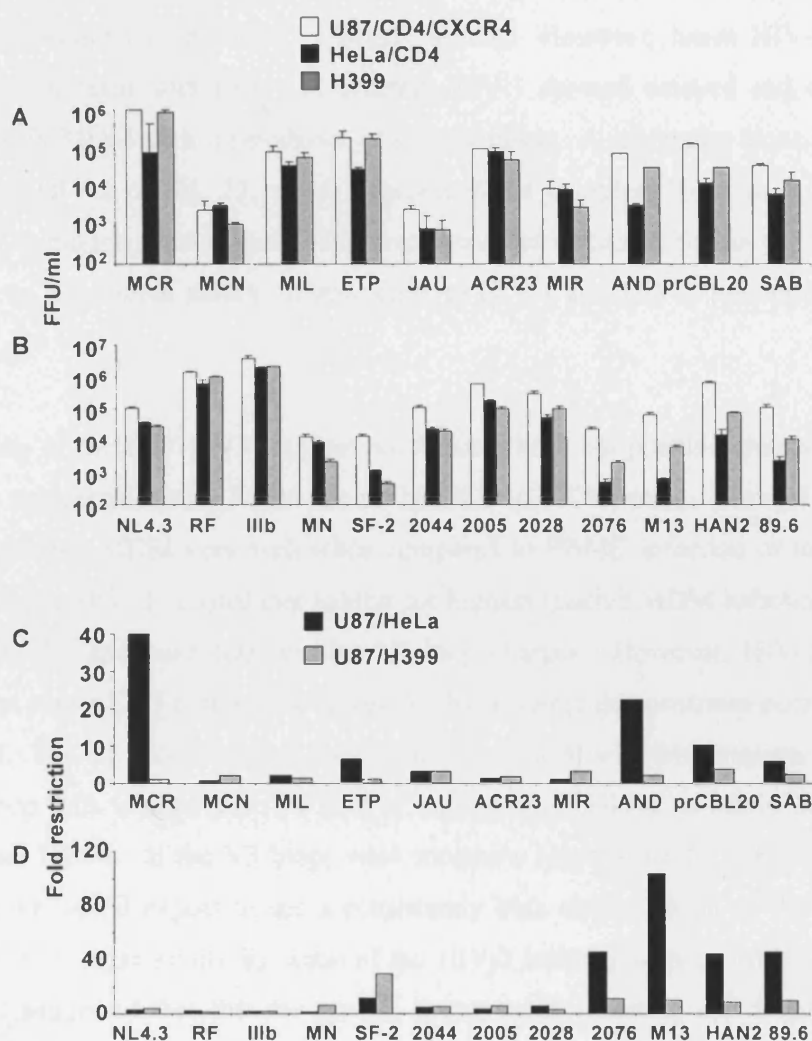


Figure 3.10. HIV-1 and HIV-2 isolates are susceptible to Lv2 restriction.

(A) An array of HIV-2 and (B) HIV-1 isolates was titrated on permissive U87/CD4/CXCR4, H399*, and restrictive HeLa/CD4 cells and the FFU/ml calculated. Fold restriction was calculated for (C) HIV-2 and (D) HIV-1 by dividing the titre on permissive U87/CD4/CXCR4 cells by the titre on restrictive HeLa/CD4 or permissive H399 cells. *H399 cells, described in greater detail in chapter 4, are a HeLa/CD4 cell line that expresses a CD4 molecule with a C-terminal tail truncation at the membrane proximal amino acid position 399. This cell line that is not Lv2 restrictive, unlike the related HeLa/CD4, is used in chapter 4 to further characterise the cellular location of Lv2 restriction.

3.3 Discussion

The data presented here show that apart from a single burst of viral replication HIV-2 continuous replication in MDM is largely absent. However, latent HIV-2 provirus could be stimulated with LPS. In contrast, HIV-1 showed delayed and rebounding replication in MDM with no evidence of a latent phase. A molecular clone, MCR was made from HIV-2 prCBL 23, which demonstrated a complete block to replication in MDM. The models used to study HIV post-entry restrictions in human cells, Lv2, was found to be a common pattern of restriction to HIV-1 and HIV-2 infection in human cells.

Sequencing of the HIV-2 V3 loop revealed that a high net positive charge of the V3 loop was associated with CXCR4 use on NP2/CD4/CXCR4 cells. None of the HIV-2 isolates infected MDM very well when compared to PBMC infection or to HIV-1 on MDM, but the HIV-2 isolates that exhibit the highest relative MDM infection are those that use CCR5 and have less positive V3 loop charges. However, HIV-2 MLC, an isolate that uses CCR5 and has the lowest V3 loop charge demonstrates poor infectivity on MDM. The V3 loop charge is only weakly correlated with MØ tropism, so overall, the V3 loop fails to determine the lack of replication of HIV-2 in MDM compared to PBMC and HIV-1. If the V3 loops were somehow responsible for the lack of MDM infection we would expect to see a consistently high charge on all of the HIV-2 V3 loops and a stronger ability by some of the HIV-2 isolates to infect MDM. A recent study has suggested that the *env* genetic determinants dictating cell tropism of dual tropic HIV-1 viruses are “complex”; they include regions of the V1/V2 and V5 hyper variable regions, and charged and uncharged amino acids in the V3 loop (Ghaffari et al., 2005), so too, the HIV-2 *env* determinants of tropism may be complex as well.

Low positive and high positive HIV-2 V3 loop charges have been reported to be associated with slow/low and fast/high viral phenotypes, respectively (Albert et al., 1996). It has also previously been reported that shift in the coreceptor usage from CCR5 to CXCR4 is associated with an increase in the net positive charge in the V3 region of HIV-2 molecular clones (Isaka et al., 1999). Here I confirm that an increasing net positive charge of the V3 loop correlates with CXCR4 usage of HIV-2 primary isolates and that there is a weak association between CCR5 using HIV-2 isolates and MØ tropism. There was a broad range of V3 loop charges among the HIV-2 isolates so it does not appear as though V3 loops alone are responsible for the lower HIV-2 TCID₅₀s in MDM compared to HIV-1.

The lower infectivity of HIV-2 compared with HIV-1 in MDM can be attributed to at least 2 factors: HIV-1 is more infectious when produced from MDM than PBMC and HIV-2 reverts to a latent state in MDM. HIV-2 primary isolates enter MDM and reverse transcribe to the same levels as seen in PBMC. However, when later time points were analysed they showed significantly less *gag*-LTR product that suggests there is less viral spread in culture or that this is just 'left-over' *gag*-LTR product from what could be seen at earlier time points post-infection.

Latency has been demonstrated for HIV-1, Jordan et al. show that the inducible replication of latent integrated HIV-1 in the T cell line Jurkat (Jordan et al., 2003). They show that a small proportion of HIV-1 integrates into or near aliphoid repeats in heterochromatin and the natural suppression of transcription in this region of the genome inhibits integrated HIV-1 transcription (Jordan et al., 2001). I have shown stimulation of HIV-2 production in MDM with LPS. The levels of stimulation of latently HIV-1 infected T cell lines has been demonstrated to vary with different agonists, particularly those that best stimulate NF- κ B, like the phorbol ester, TPA (Jordan et al., 2003). Strong NF- κ B agonists (LPS) stimulate HIV-2 replication in MDM to the same levels that were demonstrated at day 2 in HIV-2 infected MDM.

MØs have been demonstrated to be a stable source of HIV-1 replication due to their greater ability to survive virus replication whereas HIV-1 infected PBMC have been shown to readily undergo apoptosis and necrosis, several mechanisms have been

proposed (Meylan et al., 1998; Wang et al., 2001; Zhang et al., 2001) reviewed in (Alimonti et al., 2003). Gartner et al. first demonstrated that ex-vivo MØs can be infected with HIV-1, and may continue producing virus for more than 40 days (Gartner et al., 1986). Certain factors have been shown to promote the survival of MØs infected with HIV-1. Garaci et al. have shown that Nerve Growth Factor (NGF) is an autocrine factor that inhibits apoptosis and is up regulated by HIV-1 infected MØs (Garaci et al., 1999).

The *in vitro* experiments conducted here in ex-vivo MDM may have implications for disease progression in the host. HIV-2 RNA production can be induced in MDM by lipopolysaccharide five days post-infection suggesting that MDM remain infected and that provirus remains integrated at later time points. Eitner et al. have demonstrated in HIV-2 infected pig-tailed macaques that there is a highly disseminated viral phase followed by exclusive localisation of infection to the FDC network with few infected cells (Eitner et al., 2000). These findings may reflect the burst of viral RNA that I see in HIV-2 infected MDM followed by latent/loss of HIV-2 replication. Perhaps the initial burst of replication helps the virus to establish itself in the host and the subsequent latency may allow HIV-2 to avoid the immune system.

Individuals infected with HIV-2 have lower plasma viral loads than those infected with HIV-1, which reflects the lesser pathogenicity of HIV-2 (Gottlieb et al., 2002; Popper et al., 2000; Popper et al., 1999; Shanmugam et al., 2000). However the lower viral load does not equate to a lower proviral load than HIV-1 (Ariyoshi et al., 1996; Berry et al., 1994; Sarr et al., 1999), but to less viral production from the proviral template (Popper et al., 2000).

Lv2 restriction of HIV infection was introduced in this chapter. In the next chapter I demonstrate that an endocytic pathway delivers HIV to Lv2 restriction in HeLa/CD4 cells.

Chapter 4

An endocytic route of delivery to Lv2 restriction

4.1 Introduction:

In the last section, chapter 3, the restricted replication of HIV-2 isolates in MDM was identified. In particular, prCBL 23 demonstrated no replication in MDM. The molecular clone, MCR, derived from prCBL 23 virus swarm was made by my colleague Cristian Schmitz, and a restriction model using HeLa/CD4 cells as the restrictive cell type was developed (Schmitz et al., 2004). This chapter investigates the route of entry taken by Lv2 restricted and unrestricted viruses.

There have been many reports on the involvement of endocytosis in HIV infection (Fredericksen et al., 2002; Marsh and Pelchen-Matthews, 2000; Vidricaire et al., 2003; Wei et al., 2005), but so far none that have linked retroviral restriction to a particular endocytic pathway. Studying the pathways of restriction in the host cell can help to understand the pathway of infection and the preferred route of entry. It may not be the entry pathway that is restricting viral entry, per-se, but an anti-viral protein/ saturable restriction factor (Stremlau et al., 2004; Towers et al., 2000).

Clathrin mediated endocytosis was the first mechanism of receptor-mediated cellular internalisation to be discovered as a means of yolk protein uptake in the mosquito oocyte (Roth and Porter, 1964). The “archetypical” cargo to be trafficked by this pathway is the Trf receptor that internalises iron-binding protein, Trf (Harding et al., 1983; Pearse, 1982), and is commonly used as a marker of clathrin activity (Le Roy and Wrana, 2005). Sometimes called the “classical route” of endocytosis clathrin trafficking is thought to be the most active route for receptor-mediated internalisation. Once the clathrin coated pit has budded from the PM it fuses with an early endosome that can become an acidified late-endosome of pH 5.0 to 6.5.

Virus Envs can cause host-virus membrane fusion by pH-dependent and independent mechanisms. The best understood mechanism of pH-dependent fusion is that of the

influenza Haemagglutinin protein (HA), a type I fusion protein. The HA protein is folded into a high-energy state that is released upon exposure of the virion to a low pH (5.0-6.5) endosomal compartment, exposing the N-terminal fusion peptide (Wiley and Skehel, 1987). A chain-direction reversal occurs at the amino terminal end of the central coiled coil resulting in the extension of the fusion peptide towards the target membrane (Colman and Lawrence, 2003). A second chain-direction reversal occurs in the central coiled-coil region, this time projecting the carboxyl terminus back towards the amino terminus. This folding of the fusion domain back to the membrane anchor acts to bring the viral and target membranes into close apposition for fusion.

Most retroviruses and paramyxoviruses fuse with host target membranes at neutral pH and so it is the receptor binding interaction that triggers the fusion mechanism (above).

Clathrin endocytosis has been reported as an entry route for pH-dependent and independent viruses alike. Since the clathrin endocytic route can lead to an acidified compartment it is the preferred route of entry by many pH-dependent viruses: Semliki, an alphavirus, was the first confirmed pH-dependent virus to use clathrin as its primary route of entry (Helenius et al., 1980; Helenius et al., 1982; Marsh et al., 1982; White et al., 1980). Vesicular Stomatitis Virus (VSV) has also been shown to use clathrin endocytosis as its route of entry by inhibition with amantadine (Schlegel et al., 1982), and has been confirmed recently with dominant negative mutants (Sun et al., 2005). pH-dependent viruses such as Influenza, Sindbis, and human rhinovirus 14 (DeTulleo and Kirchhausen, 1998) also use this pathway of entry. HIV-1, a pH-independent virus has been shown to require clathrin, at least in part, as a route of entry (Daecke et al., 2005; Stein et al., 1987).

Caveolae were first described as “omega-like” structures in early morphology studies of the PM (Palade, 1961) and have since been shown to require the scaffolding protein caveolin to support their shape and function (Rothberg et al., 1992). Caveolin binds cholesterol and it is therefore thought that caveolae mediate cholesterol storage and homeostasis (Johannes and Lamaze, 2002; Rothberg et al., 1992), and numerous signalling and trafficking events (Anderson, 1998; Simons and Toomre, 2000). They are expressed on fibroblasts, smooth muscle cells, endothelial, and epithelial cells but not T cells (Scherer et al., 1997).

One of the few and best characterised intracellular parasites that enter via caveolae is the SV40 polyoma virus. Fewer viruses have been found that use this internalisation mechanism as a portal of entry compared to clathrin endocytosis (Sieczkarski and Whittaker, 2002a), perhaps because it is a slowly internalising, non-constitutive pathway and is therefore less active than clathrin endocytosis (Thomsen et al., 2002). This may be why SV40 requires ligand triggering of caveolae to induce actin rearrangements and uptake (Pelkmans et al., 2002). The study of SV40 internalisation has also revealed a novel organelle called the caveosome (Pelkmans et al., 2001), used as a trafficking intermediate on its way to the ER and nucleus.

Apart from clathrin-mediated and caveolae endocytosis, internalisation of cell-surface markers independently of clathrin-coated pits has led to the definition of a new type of endocytic mechanism, clathrin independent endocytosis (Nichols and Lippincott-Schwartz, 2001). Internalising lipid (Puri et al., 2001) and protein (Nichols et al., 2001) markers have been visualised outside of clathrin pits suggesting that clathrin independent routes exist, but also in different compartments suggesting the existence of numerous non-classical routes of endocytosis (Nichols and Lippincott-Schwartz, 2001). The membrane organisation of vesicles in these pathways is thought to be composed, primarily of lipid rafts (Simons and Ikonen, 1997), and the organisation of some lipid raft markers post-internalisation, such as GPI-linked proteins, is thought to occur in discreet compartments independent of the classical early endosome antigen 1 (EEA1) endosome (Sabharanjak et al., 2002). Some lipid raft markers such as the M2 muscarinic acetylcholine receptors internalise independently of clathrin but traffic into a classical clathrin/Rab5 positive early endosome post-internalisation (Delaney et al., 2002).

Virus entry by clathrin/caveolae independent pathways is highly variable and obligate in some cases. Murine polyomavirus (MPV) requires actin depolymerisation and stable microtubules for infection and a clathrin/caveolae independent pathway for productive entry (Gilbert and Benjamin, 2000; Gilbert et al., 2003). Similar to MPV, SV40 can use at least one caveolae/clathrin independent route of entry in addition to caveolae (Damm et al., 2005). Clathrin independent uptake via “smooth surfaced pits and vesicles” was implicated in early studies of influenza virus entry (Matlin et al., 1981). The use of

dominant negative mutants has confirmed influenza virus entry by both clathrin and clathrin/caveolae independent endocytosis in the same cell (Lakadamyali et al., 2004; Rust et al., 2004; Sieczkarski and Whittaker, 2002b). Even though influenza does not require clathrin for initial entry into the cell it does require the classical Rab 5 and Rab 7 acidified, late endosomal compartments for productive infection (Sieczkarski and Whittaker, 2003).

Depletion of membrane cholesterol (using cyclodextrins) has shown that PM lipid rafts are necessary for HIV-1 binding and entry (Liao et al., 2001), and may be required for clustering of CD4 receptor and coreceptors to sites of virus binding (Manes et al., 2000). However, truncation of the CD4 cytoplasmic domain suggests that CD4 does not need to be localised to lipid rafts for efficient HIV-1 entry and infection (Bedinger et al., 1988; Popik and Alce, 2004). The involvement of lipid rafts in HIV entry may occur post-CD4 binding by destabilisation of lipid raft domains (Kozak et al., 2002). It has even been suggested that virion associated lipid rafts are required for virus infectivity (Graham et al., 2003; Liao et al., 2003).

4.2 Results:

4.2.1 MCR is not restricted when fusion is forced at the cell surface.

MCR can be rescued in HeLa/CD4 cells when it is pseudotyped with VSV-G (fig 4.1a)(Schmitz et al., 2004). VSV-G is a pH-dependent Env that requires delivery into an acidified endosome compartment to trigger fusion with the host cell (Blumenthal et al., 1987). Since MCR avoids restriction by delivery into an endosomal pathway with a VSV Env, I determined if MCR restriction could be overcome if MCR delivery was forced, outside of an endosomal compartment, at the cell surface with a VSV Env. The fusion of VSV-G at the cell surface by transient acid-shock with acidified (pH 5.5) media has been described previously (Marsh and Bron, 1997). The buffering of growth media to a pH of 5.0-5.5 emulates the pH of late endosomes and acts to fuse pH-dependent Envs at the cell surface when added transiently to pH-dependent-virus and cell mixtures (Marsh and Bron, 1997; White et al., 1980).

Fig 4.1a and b show a comparison of MCR and MCR(VSV). Infection of MCR is rescued if restrictive cores are pseudotyped with VSV Env (fig 4.1a). MCR infection in HeLa/CD4 cells is not affected by monensin neutralisation of endosomal pH (control) or by the transient acid treatment at pH 5.5 compared to normal infection. However infection is recovered more than 10-fold when MCR(VSV) is forced to fuse at the cell surface by transient acid shock ('pH 5.5 + monensin') (fig 4.1b). When VSV-G mediated fusion is prevented by monensin it fails to rescue MCR [MCR (VSV) 'pH 6.8 + monensin', fig 4.1b].

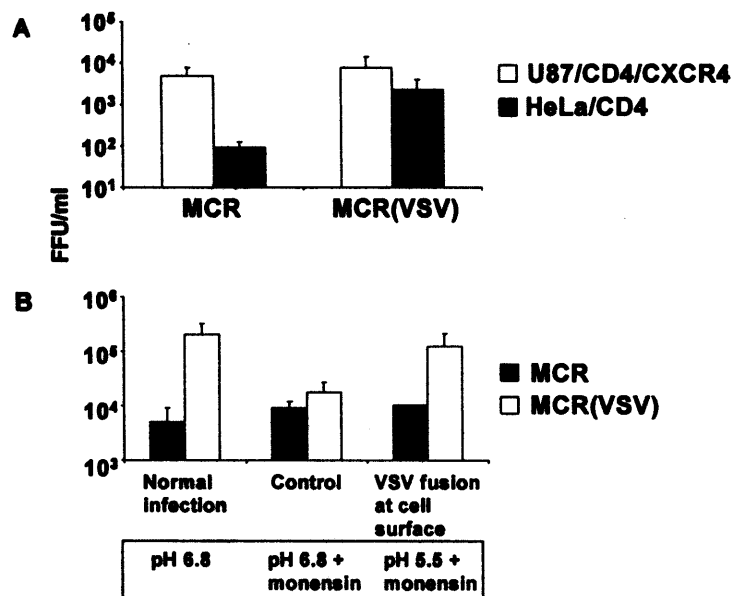


Figure 4.1. MCR pseudotyped with vesicular stomatitis virus envelope is rescued from Lv2 restriction in HeLa/CD4 cells: MCR is not restricted in HeLa/CD4 cells when forced to fuse at the cell surface.

(A) MCR and MCR(VSV) were titrated on U87/CD4/CXCR4 and HeLa/CD4 cells and fixed and stained for HIV-2 infection 3 days later. (B) Surface fusion of MCR(VSV) by transient acid shock. MCR and MCR(VSV) were titrated on HeLa/CD4 cells and treated with low pH media for 2 min to induce fusion (pH 5.5 + monensin) or controls with physiological pH media with or without monensin ('pH 6.8 + monensin' and 'pH 6.8', respectively), low pH media was replaced with pH 6.8 buffered growth media and cells were fixed and stained for HIV-2 infection 3 days later (materials and methods). The results shown are representative of at least 4 independent experiments. Error bars represent the SDM.

4.2.2 Lv2 restriction does not require acidified endosomes to inhibit virus entry.

Bafilomycin A1 is a lysosomal tropic agent that neutralises the pH of late endosomes and lysosomes, effectively inhibiting the fusion and entry of pH-dependent viruses (Drose et al., 1993; Helenius et al., 1982; Marsh et al., 1982; Perez and Carrasco, 1993). Previous reports have demonstrated that HIV-1 does not require a low pH for fusion (McClure et al., 1988; Stein et al., 1987). I determined the effect of pH on MCR restriction in HeLa/CD4 cells. Fig 4.2 shows that treatment of HeLa/CD4 cells with bafilomycin A1 can inhibit infection by a pH-dependent VSV Env [MCR(VSV)] by more than 99%. Bafilomycin does not alter the infectivity of MCR on restrictive HeLa/CD4 cells (fig 4.2). Interestingly, MCR infectivity decreased (by approximately 3-fold) from a mean of 145 to 45 FFU on permissive U87/CD4/CXCR4 cells with bafilomycin treatment. Though there was no effect on infection in HeLa/CD4 cells there was a decrease in restriction from 11-fold to 3-fold, upon bafilomycin treatment. This was due entirely to a drop in infection of MCR on permissive U87/CD4/CXCR4 cells and not any change in infection on restrictive HeLa/CD4 cells (fig 4.2).

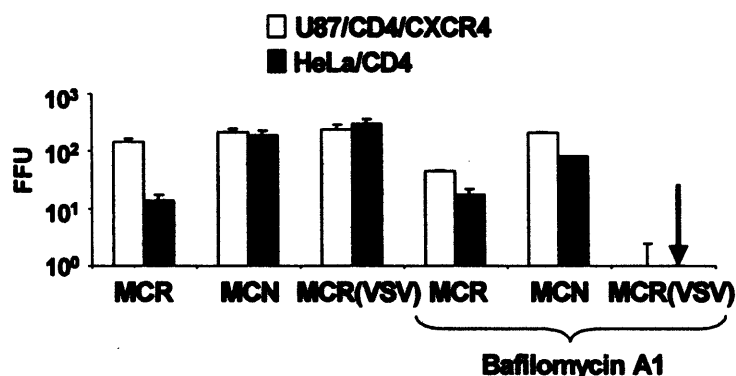


Figure 4.2. Neutralisation of intracellular pH with bafilomycin A1 doesn't affect MCR restriction in HeLa/CD4 cells.

Viruses and pseudotypes were plated on U87/CD4/CXCR4 and HeLa/CD4 cells in the presence of bafilomycin A1. Cells were washed with normal growth media and infection was allowed to proceed in the absence of bafilomycin A1. Cells were fixed and stained for HIV-2 infection 3 days later. Results shown are representative of 3 independent experiments. Error bars represent SDM.

4.2.3 Hypertonic media inhibit endocytosis of transferrin and cholera toxin binding subunit in U87/CD4/CXCR4 and HeLa/CD4 cells.

I determined the effect of endocytosis inhibition on restriction in HeLa/CD4 cells, without affecting the pH of endosomal compartments. Hypertonic sucrose inhibits receptor-mediated endocytosis without affecting pH (Daukas and Zigmond, 1985; Heuser and Anderson, 1989). As a positive control for inhibition of endocytosis I used FITC labelled Trf (Trf-FITC)(fig 4.3a-d), which is internalised by clathrin coated pits (Gruenberg, 2001), and FITC labelled CTxB (CTxB-FITC)(fig 4.3e-h) which is internalised by a lipid raft dependent pathway (Wolf et al., 2002). Hypertonic sucrose inhibited Trf-FITC and CTxB-FITC uptake in both the restrictive HeLa/CD4 and permissive U87/CD4/CXCR4 cells, signified by capping of FITC at the cell surface (fig 4.3b, d, f, h).

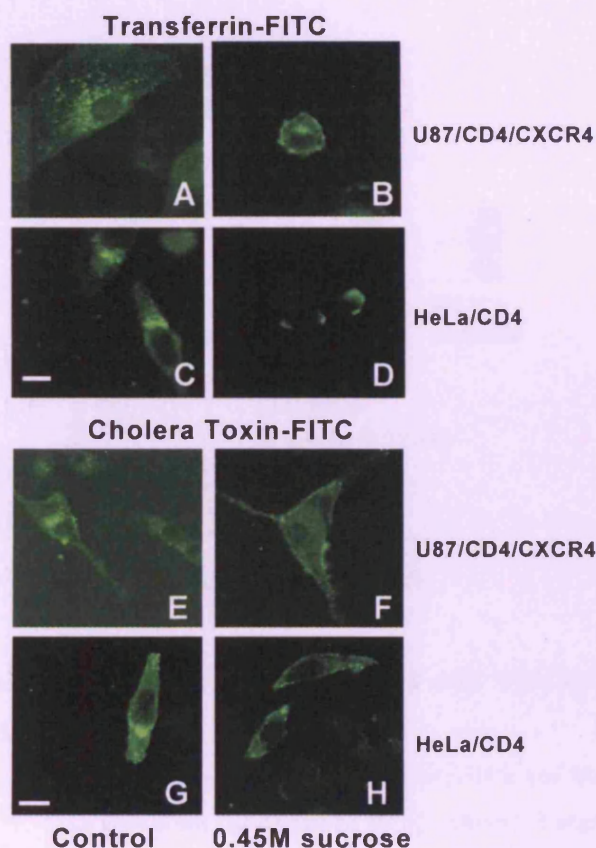


Figure 4.3. Sucrose inhibits transferrin and cholera toxin-B uptake.

Transferrin-polylysine-FITC (a-d) or cholera toxin binding-subunit-FITC (e-h) were bound to the permissive U87/CD4/CXCR4 or restrictive HeLa/CD4 cells with or without 0.45M sucrose and examined by confocal microscopy. Inhibition is shown by Trf-FITC or CTxB-FITC capping at the cell surface with

simultaneous uptake into perinuclear (endosomal) regions of the cell. White scale bar indicates 10µm magnification. Results are representative of at least 4 independent experiments.

4.2.4 Lv2 restriction depends on an endocytic pathway of entry that is directed by the viral envelope.

Fig 4.4 shows the effect of hypertonic sucrose on restricted MCR infection. Unrestricted MCN and restricted MCR were plated on sucrose treated HeLa/CD4 and U87/CD4/CXCR4 cells. The infectivity of MCR on HeLa CD4 cells increased 6.5-fold from a mean of 16 to a mean of 105 FFU with sucrose treatment (fig 4.4a). Fig 4.4b shows the resultant fold restriction decrease from 12.0-fold to 1.5-fold. There was no effect of sucrose treatment on MCR infection of permissive U87/CD4/CXCR4 cells or on MCN infection of either HeLa/CD4 or U87/CD4/CXCR4 cells (fig 4.4a).

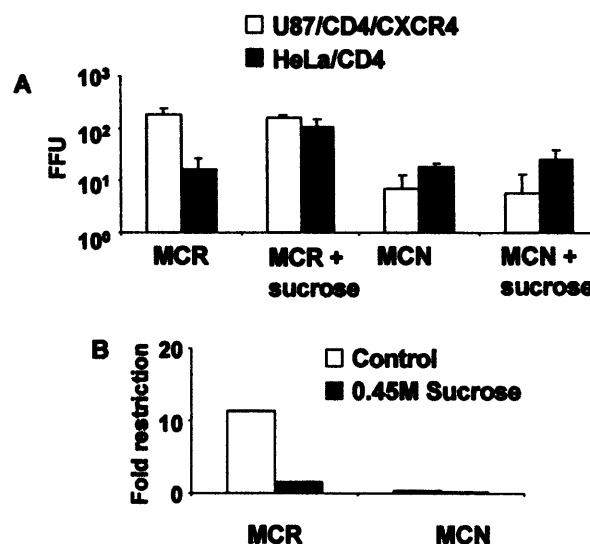


Figure 4.4. Treatment of Lv2 restrictive HeLa/CD4 cells with hypertonic sucrose recovers restricted MCR infection.

Cells were plated with a U87/CD4/CXCR4 standardised dose of (A) MCR and MCN and treated with hypertonic sucrose for 1 hr. Cells were fixed and stained for HIV-2 infection 3 days later. (B) The FFU from permissive U87/CD4/CXCR4 infection were divided by the infection on restrictive HeLa/CD4 cells to obtain the fold restriction, for control and 0.45M sucrose treatments. Results are representative of 5 independent experiments. Error bars represent SDM.

Lv2 restriction maps to both the viral *env* and *gag* genes (Schmitz et al., 2004). I determined whether the rescue of restriction with hypertonic sucrose was due to *env* or *gag* or both. Fig 4.5b shows the restriction of MCN containing a restricted Env (MCNmcrenv) is rescued from 21.0-fold to 1.0-fold, in this experiment, with hypertonic sucrose treatment. The MCN molecular clone with a restricted Gag (MCNmcr^{gag}) was less restricted but was completely rescued from 4.0-fold to 1.0-fold of restriction (fig 4.5b). Therefore both the *env* and *gag* mediated restrictions of Lv2 are rescued by inhibition of endocytosis.

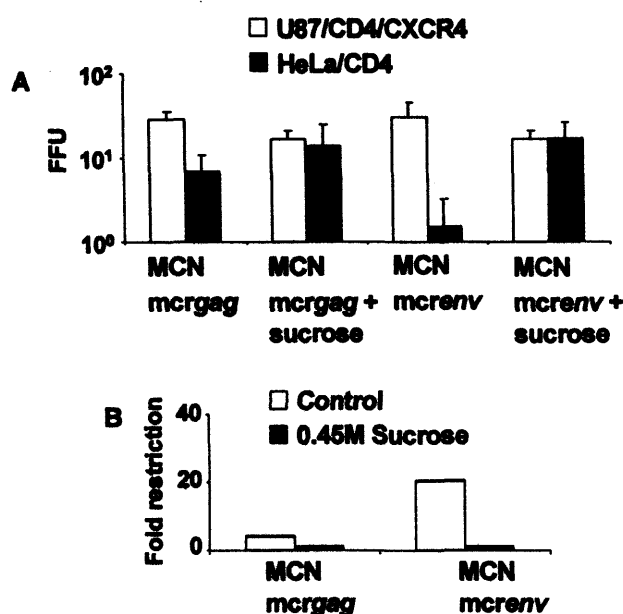


Figure 4.5. Recovery of MCR infection with hypertonic sucrose is mediated by both *gag* and *envelope*.

(A) Cells were titrated with MCNmcr^{gag} and MCNmcrenv viruses and treated with hypertonic sucrose. Cells were fixed and stained for HIV-2 infection 3 days later. (B) The permissive U87/CD4/CXCR4 infection was divided by the FFU on restrictive HeLa/CD4 cells to obtain the fold restriction. Results are representative of 3 independent experiments. Error bars represent SDM.

4.2.5 Infectivity of HIV-1 retroviral vectors pseudotyped with MCR and MCN envelopes in HeLa/CD4 cells can be improved by inhibition of endocytosis.

HIV-1 retroviral p8.91 particles pseudotyped with MCR and MCN Envs were plated on HeLa/CD4 and U87/CD4/CXCR4 cells treated with hypertonic sucrose. Fig 4.6a shows that the infectivity of both the MCR and MCN vector pseudotypes was rescued in sucrose treated HeLa/CD4 cells by 8.5 and 5.0-fold, respectively. Sucrose treatment had little effect on the infectivity of either of the HIV-2 Env pseudotypes on U87/CD4/CXCR4 cells (fig 4.6b).

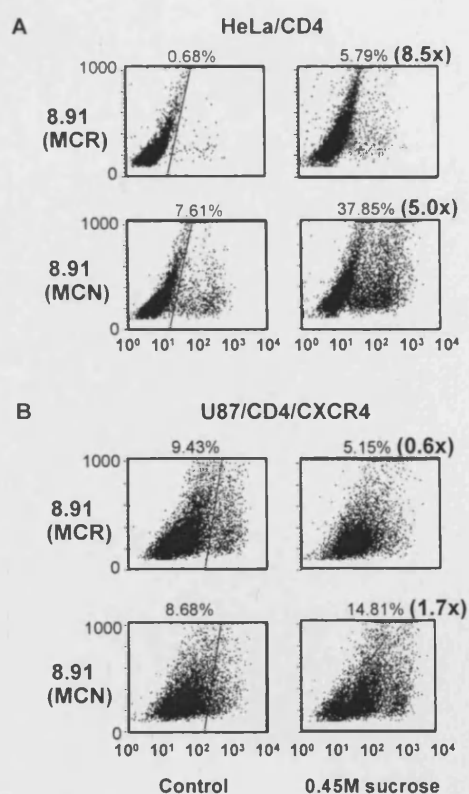


Figure 4.6. HIV-1 vector pseudotypes with MCR and MCN envelopes are rescued by treatment with hypertonic sucrose in restrictive HeLa/CD4 cells.

(A) HeLa/CD4 and (B) U87/CD4/CXCR4 cells were plated with a U87/CD4/CXCR4 standardised dose of HIV-1 vector pseudotypes HIV-1_{8.91}(MCR) and HIV-1_{8.91}(MCN) and treated with hypertonic sucrose for 1 hr. EGFP positive cells were determined by FACS three days post-infection. Results shown are representative of 2 independent experiments.

4.2.6 Depletion of membrane cholesterol with methyl- β -cyclodextrin inhibits uptake of cholera toxin binding subunit: membrane trafficking differences between restrictive and permissive cells.

Membrane cholesterol is required for the endocytic trafficking of lipid rafts (Sandvig et al., 2004). I determined the effect of membrane cholesterol depletion on Lv2 restriction using M β CD. As controls, the disruption of lipid rafts by M β CD was followed in HeLa/CD4 and U87/CD4/CXCR4 cells with CTxB-FITC and Trf-FITC. Cholera toxin trafficking is via a lipid raft dependent endocytic pathway that is disrupted by depletion of PM cholesterol (Fujinaga et al., 2003; Nichols, 2002; Sandvig et al., 2004; Sandvig and Van Deurs, 2002). Fig 4.7d, e, f show that internalisation of CTxB in HeLa/CD4 cells is inhibited by M β CD and can be reconstituted with water-soluble cholesterol. However, the internalisation of CTxB-FITC in U87/CD4/CXCR4 cells was not inhibited by treatment with M β CD (fig 4.7a, b, c). As expected internalisation of clathrin dependent Trf-FITC was not inhibited by depletion of membrane cholesterol with M β CD (fig 4.7g-j).

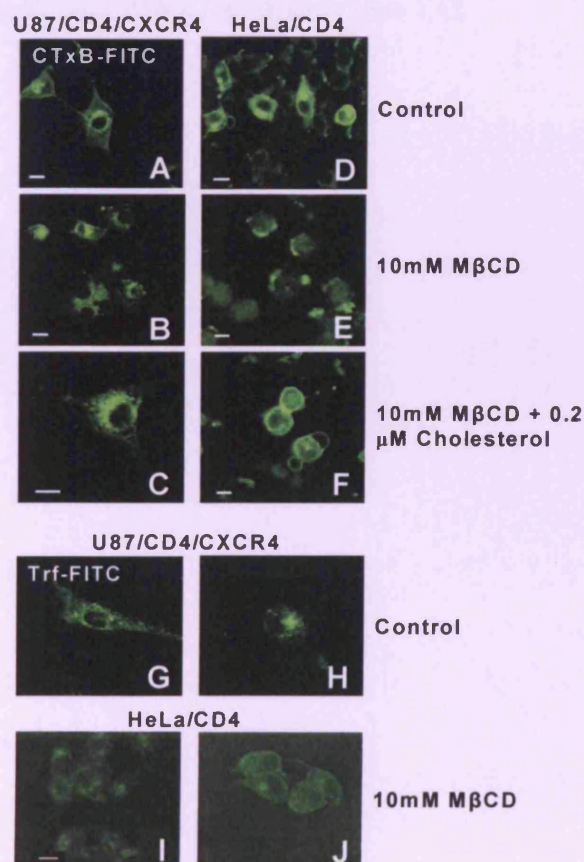


Figure 4.7. Methyl- β -cyclodextrin differentially inhibits cholera toxin-B uptake in Lv2 restricted and permissive cells.

Permissive U87/CD4/CXCR4 or restrictive HeLa/CD4 cells were treated with M β CD. CTxB-FITC (a-f) and Trf-FITC (g-j) were bound to treated or untreated cells for 1hr with or without water-soluble cholesterol and examined by confocal microscopy. Capping of CTxB-FITC at the cell surface shows inhibition. White scale bar indicates 10 μ m magnification. Results are representative of at least 4 independent experiments.

MCR, MCN, MCNmcr gag , and MCNmcr env viruses were plated on M β CD treated HeLa/CD4 and U87/CD4/CXCR4 cells. The titre of all viruses was slightly decreased on U87/CD4/CXCR4 cells (fig 4.8b). Fig 4.8a shows that MCR titre on restricted HeLa/CD4 cells increased 8.0-fold with M β CD treatment from a mean of 15 to a mean of 120 FFU and restriction could be partially reconstituted to a mean of 25 FFU by adding back water-soluble cholesterol. The unrestricted MCN was not affected by any of these treatments on HeLa/CD4 cells. The titres of MCNmcr env and MCNmcr gag on HeLa/CD4 cells were not affected by M β CD treatment (data not shown). M β CD had no effect on the infectivity of a VSV Env pseudotype in fig 4.8c, supporting the evidence that the pathway delivered to by VSV-G is different from Lv2.

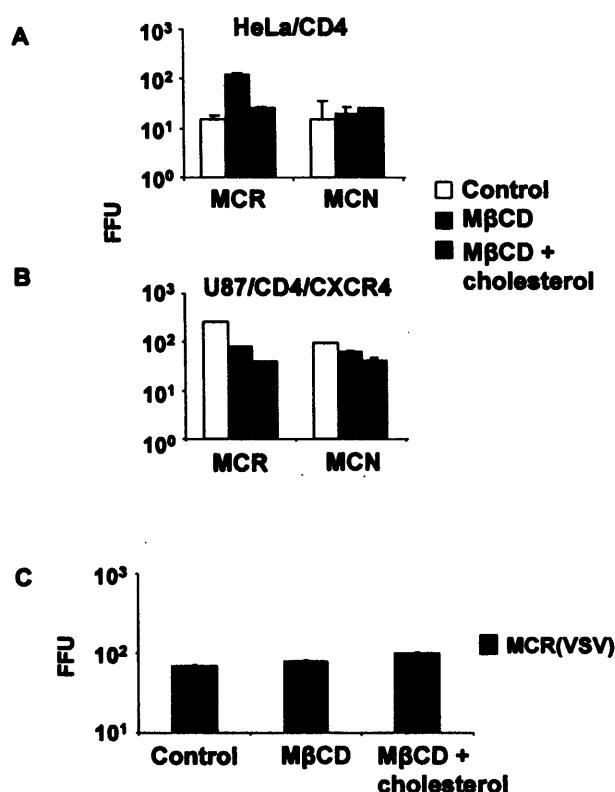


Figure 4.8. MCR infection in restrictive HeLa/CD4 cells is rescued by treatment with methyl- β -cyclodextrin: MCR *gag* and *envelope* are both required for recovery of infection by cholesterol depletion.

(A) HeLa/CD4 and (B) U87/CD4/CXCR4 cells were treated with 10mM M β CD for 30 min at 37°C, fixed doses (standardised on U87/CD4/CXCR4) of viruses were added on ice for 1 hr, and cells were incubated in serum free media +/- 0.20 μ M cholesterol at 37°C for 2 hr. Growth media was added and cells were fixed and stained for HIV-2 infection 3 days later. (C) Cholesterol depletion does not affect entry of VSV Env on HeLa/CD4 cells. The results shown are representative of at least 6 independent experiments. Error bars represent the SDM. The titres of MCR and MCN on HeLa/CD4 cells are similar in (A) because input doses were equalised for ease of comparison.

4.2.8 Directing CD4 out of lipid rafts rescues restricted virus infection.

CD4 localises primarily within lipid rafts on the PM (Popik et al., 2002). I used a HeLa cell line expressing a mutant CD4 with a stop codon at amino acid position 399 (H399) in the cytoplasmic tail (fig 4.9a). The locations of wild-type CD4 compared to CD4_{H399} within lipid rafts were verified. Lipid rafts were prepared from U87/CD4/CXCR4,

HeLa/CD4, and H399 cells on a sucrose gradient and densitometry was performed on the fractions to determine the precise quantity of CD4 in lipid rafts. The lipid raft marker placental alkaline phosphatase (PLAP) was tested in parallel for each fraction. Fig 4.9b shows that the CD4 of U87/CD4/CXCR4 and HeLa/CD4 cells is found predominantly in the lipid raft fractions 3,4, and 5 as is the lipid raft marker, PLAP in fig 4.9c. In contrast, the CD4 of H399 cells is found primarily in fractions 9 and 10 and was thus excluded from lipid rafts. C-terminally truncated CD4 localising outside of lipid rafts has been verified (Popik and Alce, 2004). This truncation is upstream of position 419-427, the poly arginine tract, shown to direct CD4 into lipid rafts (Popik and Alce, 2004).

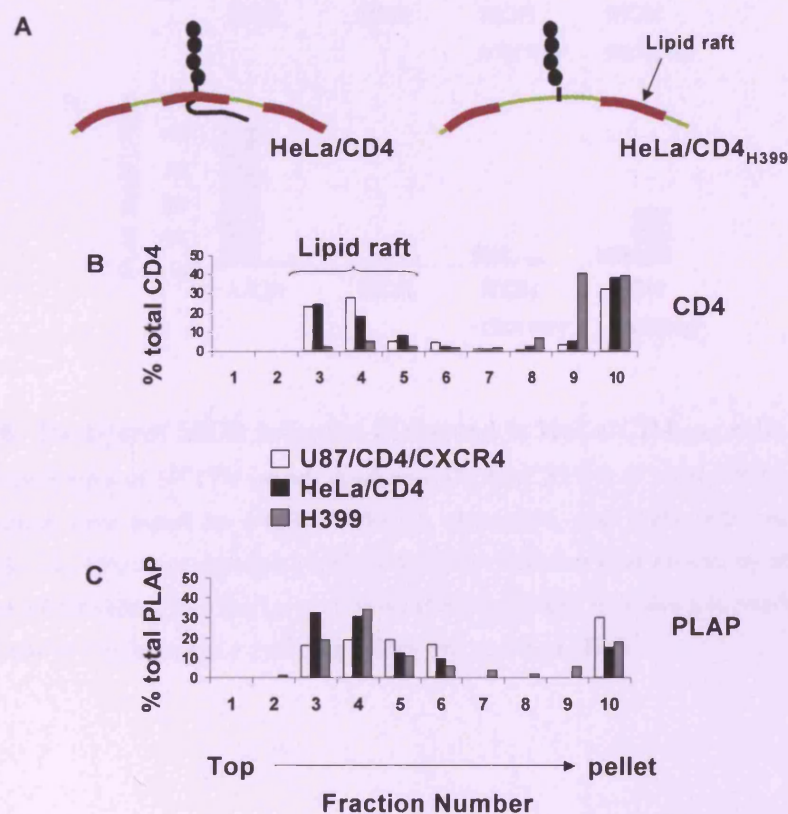


Figure 4.9. CD4 is directed out of lipid rafts by truncation of its cytoplasmic tail.

(A) Truncation of CD4 cytoplasmic tail at membrane proximal histidine 399 (H399). Lipid rafts were prepared from membrane lysates of U87/CD4/CXCR4, HeLa/CD4, and H399 and analysed for (B) CD4, and (C) placental alkaline phosphatase (PLAP) localisation as a lipid raft marker. Results are representative of 4 independent fractionations.

Fig 4.10b shows that the restriction of MCR in HeLa/CD4 cells is recovered in H399 cells from 42.0-fold to 1.4-fold. The restrictions of MCNmcrenv and MCNmcr gag were much lower than MCR, as expected (6.0 and 5.0-fold respectively). Interestingly, the clones that expressed the MCN Env demonstrated a drop in infection on H399 cells and those that expressed the MCR Env showed an enhancement of infection on H399 cells compared to HeLa/CD4 cells in all 5 independent experiments.

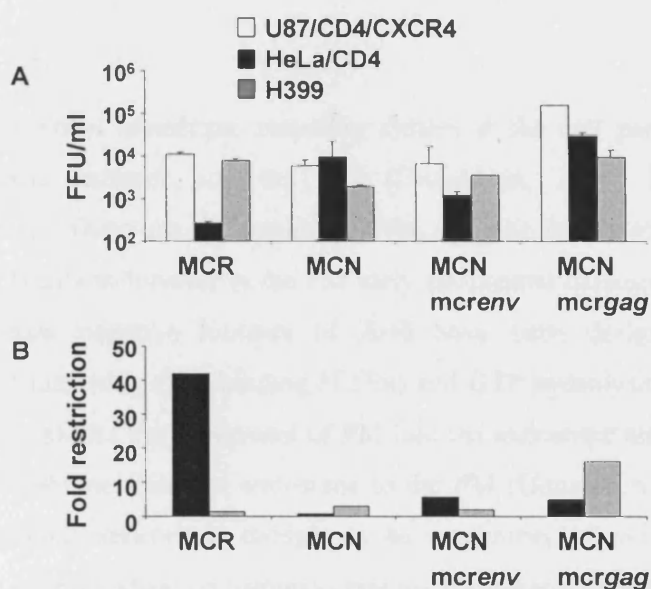


Figure 4.10. Restricted MCR infection is rescued in HeLa/CD4_{H399} cells.

(A) An equivalent input of 10⁴ FFU (standardised on U87/CD4/CXCR4) of MCR, MCN, MCNmcrenv, and MCNmcr gag were plated on U87/CD4/CXCR4, HeLa/CD4, and H399 cells and the FFU/ml calculated. (B) The FFU from permissive U87/CD4/CXCR4 infection were divided by the infection on restrictive HeLa/CD4 cells (black bars) or permissive H399 (grey bars) to obtain fold restriction. Results are representative of 5 independent experiments. Error bars represent SDM.

4.2.9 Endocytic pathways are specifically inhibited with dominant negative mutants.

Inhibition of endocytosis with M β CD is cell-type dependent and hypertonic sucrose blocks numerous pathways (fig 4.5). In some cell types, M β CD will also inhibit clathrin-mediated endocytosis (Rodal et al., 1999). Dominant negative mutants are used here to target the three major pathways of endocytosis specifically: clathrin

(Eps15)(Benmerah et al., 1999; Benmerah et al., 1998), caveolae (caveolin-1) (Pelkmans et al., 2001), and non-clathrin non-caveolae (Arf6-T27N) mediated endocytosis (Fig 4.11a)(Radhakrishna and Donaldson, 1997). Dynamin is a 'pinchase' that is used by both clathrin and caveolae endocytosis, thus the dynamin dominant negative mutant K44A will inhibit both of these pathways (fig 4.11a) (van der Blik et al., 1993), whereas the recycling endosome pathway mediated by Adenosine ribosylation factor 6 (Arf6) has been reported to be dynamin independent (Delaney et al., 2002).

Arf6 regulates a novel membrane recycling system at the cell periphery, between a tubular endosome network and the PM (Donaldson, 2003; Radhakrishna and Donaldson, 1997). There are six members of the *arf* gene family and Arf6 is the only member of the family to localise to the PM early endosomal network (Cavenagh et al., 1996). Dominant negative mutants of Arf6 have been designed to study the consequences of inhibiting GTP binding (T27N) and GTP hydrolysis (Q67L) (Peters et al., 1995). Q67L blocks the movement of PM into the endosome and T27N blocks the return of the membrane from the endosome to the PM (Donaldson, 2003). The Arf6 recycling endosomal network is thought to be a member of the non-clathrin non-caveolae group of internalisation pathways that are cholesterol dependent (Naslavsky et al., 2004).

Thus far, the Arf6 pathway has not been implicated in the entry of any virus. Although Arf6 has been implicated in the HIV-1 Nef mediated down regulation of MHC class I molecules from the cell surface (Blagoveshchenskaya et al., 2002), however the physiological relevance of this work remains disputed (Larsen et al., 2004).

The Arf6 tubular endosome recycles MHC-I from the cell surface and is involved in the trafficking of the IL-2 receptor alpha subunit (Tac). When trafficking is inhibited with the Arf6 T27N dominant negative mutant or RNAi MHC-I is directed down a degradative clathrin mediated pathway and results in less expression at the cell surface (fig 4.11b) (Naslavsky et al., 2003; Naslavsky et al., 2004). The dynamin K44A mutant and the clathrin Eps15 mutant inhibit entry of VSV into an acidified endosomal pathway for fusion and entry (Sun et al., 2005). Fig 4.11d shows that expression of these mutants knocked down VSV infection by 70% in HeLa/CD4 cells. Arf6 has been

reported to direct some endosomal traffic into low pH compartments (Delaney et al., 2002), thus there is a limited (28%) inhibition of VSV infection in the Arf6 T27N knockdown cells (fig 4.11d).

Caveolae have been implicated as an entry pathway for SV40 and (Pelkmans et al., 2001), more recently, amphotropic-MLV (MLV-A) (Beer et al., 2005). The MLV-A receptor, PiT-2 colocalises with caveolin-1, directing MLV-A into this pathway (Beer et al., 2005). N-terminally eGFP-tagged caveolin-1 (dnCav) behaves in a dominant negative fashion when transfected into cells that endogenously express caveolin-1, whereas C-terminally eGFP-tagged caveolin-1 acts in a wild-type fashion (Pelkmans et al., 2001). The dynamin K44A and dnCav1 mutants inhibited HIV(MLV-A) infection by 93 and 95%, respectively, compared to the transfection control in fig 4.11c. However, HIV-1 NL4.3 was not affected by any of these treatments (fig 4.11c).

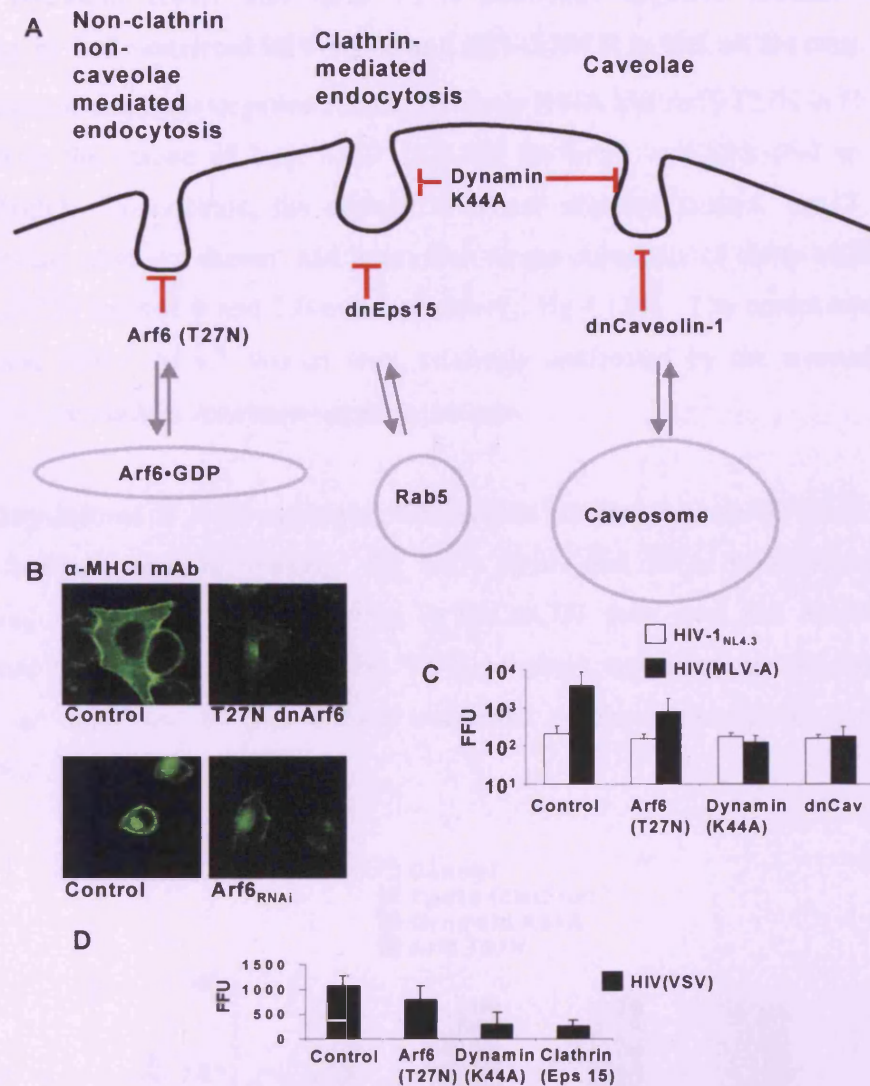


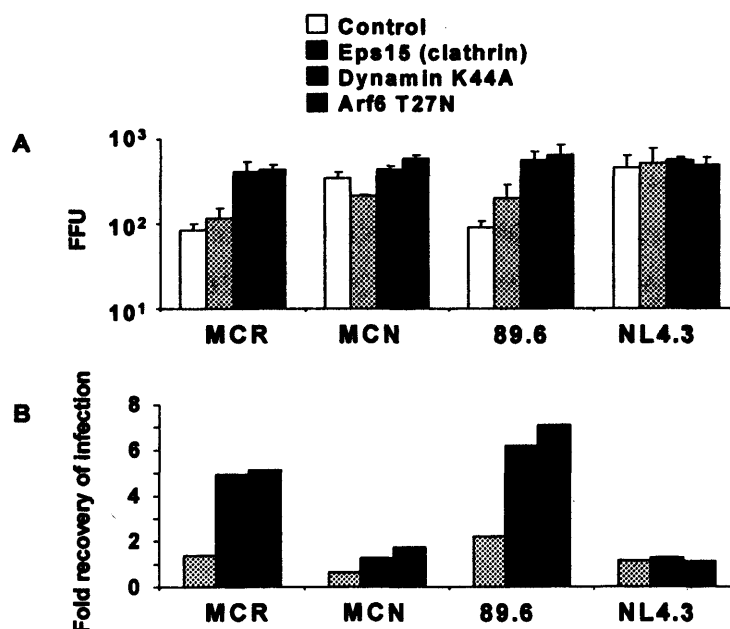
Figure 4.11. Dominant negative mutants targeted at key endocytic proteins block specific endocytic pathways.

(A) Diagram of the different endocytic pathways targeted by dominant negative mutants in this study. (B) HeLa/CD4 cells were transfected with the Arf6 T27N mutant or Arf6 RNAi for 48 hr and stained with primary MHC-I mAb and FITC conjugated secondary antibody as described in materials and methods. Cells were visualised by confocal microscopy. (C) HeLa/CD4 cells were transfected with dominant negative mutants and infected with HIV-1 NL4.3 and an amphotropic-MLV Env pseudotype of HIV-2 [HIV(MLV-A)], fixed and stained for HIV-1 and HIV-2 infection two days later. (D) HeLa/CD4 cells were transfected with control, Arf6, dynamine (K44A), and clathrin (Eps15) dominant negative mutants for 48 hr, infected with an HIV(VSV) pseudotype and fixed and stained for HIV infection 3 days later.

4.2.10 Dynamin K44A and Arf6 T27N dominant negative mutants recover infection by Lv2 restricted HIV-1 89.6 and HIV-2 MCR in HeLa/CD4 cells.

Expression of dominant negative mutants dynamin K44A and Arf6 T27N in HeLa/CD4 resulted in the rescue of both MCR (5.0-fold for both) and 89.6 (6.0 to 7.0-fold respectively). In contrast, the clathrin dominant negative mutant, Eps15 and the caveolin-1 (data not shown) had little effect on the infectivity of either MCR or 89.6 in HeLa/CD4 cells (1.0 and 2.0-fold respectively, fig 4.12b). The unrestricted HIV-2 MCN and HIV-1 NL4.3 viruses were relatively unaffected by the dynamin, Arf6, caveolin-1, or clathrin dominant negative mutants.

RNAi knockdown of Arf6 expression was used to confirm the specificity of the Arf6 T27N dominant negative mutant. Fig 4.12c shows that RNAi knockdown of Arf6 recovered 3.0-fold of MCR infection in HeLa/CD4 cells over the RNAi control, compared to 3.7-fold when the Arf6 T27N construct was used, in this experiment. MCN was unaffected by any of these treatments, confirming a role for Arf6 in Lv2 restriction.



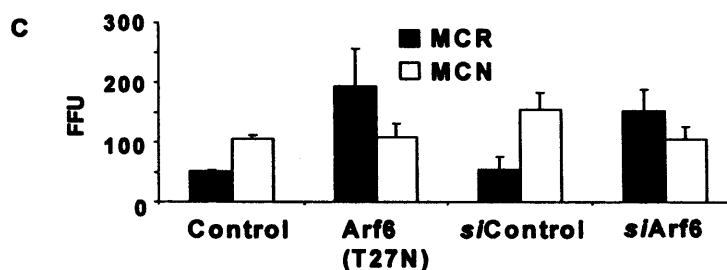


Figure 4.12. Arf6 and dynamin dominant negative mutants recover Lv2 restricted HIV-1 and HIV-2 infection in HeLa/CD4 cells.

(A) HeLa/CD4 cells were transfected with specific endocytosis knockdown mutants or (C) siRNA oligomers directed against control and Arf6, and infected two days later with titrations of restricted (A) HIV-1 89.6, (A,C) HIV-2 MCR and unrestricted (A) HIV-1 NL4.3 and (A,C) HIV-2 MCN. Control represents infection of cells transfected with an eGFP expressing construct or siRNA directed against the D6 blood group antigen. (B) Infection on cells transfected with each dominant negative mutant in (A) was divided by the infection from the control in (A) to obtain fold recovery of infection. Results are representative of 7 independent experiments. Error bars represent SDM.

4.2.11 HeLa/CD4 cells expressing Arf6 T27N and dynamin K44A dominant negative mutants rescued Lv2 restricted infection by MCR *gag* and *envelope* gene swap molecular clones.

Similar to the results in fig 4.12, the recovery of MCR with dynamin K44A and Arf6 T27N was 4.5 and 5.0-fold, respectively (fig 4.13b). The gene swap molecular clone viruses each showed about half the recovery of MCR with the Arf6 and dynamin dominant negative mutants: MCNmcr*gag* and MCNmcr*env* were rescued between 2.0 and 3.0-fold with dynamin K44A, or Arf6 T27N mutant on HeLa/CD4 cells (Fig 4.13b).

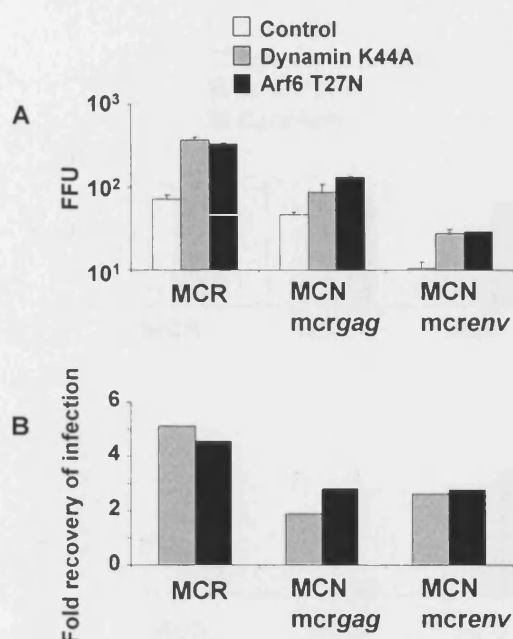


Figure 4.13. Arf6 and dynamin dominant negative mutants recover *gag* and *envelope* gene swap virus infection in HeLa/CD4 cells.

HeLa/CD4 cells were transfected with endocytosis knockout mutants and infected 2 days later with (A) MCR *gag* and *env* swaps, MCNmcrgag and MCNmcrenv, and fixed and stained for HIV-2 infection three days later. (B) Infection on cells transfected with each dominant negative mutant was divided by the infection from the control to obtain fold recovery of infection. Results shown are representative of 3 independent experiments. Error bars represent SDM.

The restriction of HIV-2 MCR and HIV-1 89.6 is recovered by inhibiting Arf6 or dynamin activity so it is interesting to see what effect knocking out both of these pathways, at the same time, has on restricted virus infection. Fig 4.14e shows that the double knockdown of Arf6 and dynamin doesn't increase recovery of infection of MCR over that seen with the single knockdowns but there is an additive effect of double Arf6/dynamain knockdown on recovery of 89.6 infection. 89.6 was recovered 2.2-fold and 4.3-fold with the dynamain and Arf6 mutants, respectively, but the recovery increased to 10.5-fold with the dynamain/Arf6 double knockout (fig 4.14b).

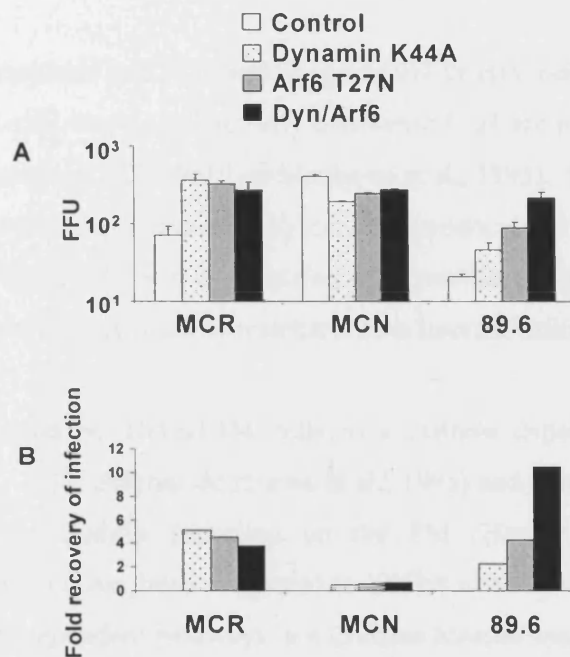


Figure 4.14. Cotransfection of Arf6 and dynamin dominant negative mutants has an additive effect on restriction recovery of HIV-1 89.6 in HeLa/CD4 cells.

(A) Arf6 T27N and dynamin K44A mutants were cotransfected into HeLa/CD4 cells (Dyn/Arf6) or individually, infected with restricted HIV-2 MCR, HIV-1 89.6, and unrestricted MCN. (B) The FFU obtained from infection on cells transfected with each dominant negative mutant was divided by the infection from the control to obtain fold recovery of infection. Results are representative of 3 independent experiments. Error bars represent SDM.

4.3 Discussion:

This chapter demonstrates that a lipid raft dependent endocytic pathway mediates Lv2 restriction in HeLa/CD4 cells. Restricted virus Env directs the viral core into this restrictive Lv2 compartment, where trafficking of the retrovirus to the nucleus is terminated. Specific pathways of endocytosis were blocked with dominant negative mutants and it was demonstrated that direction into the restrictive compartment is via an Arf6 and dynamin dependent pathway, and at least partly explains Lv2. Furthermore, the Lv2 compartment is pH-independent and the classical clathrin and caveolae endocytic pathways are not involved. These data support the model that Lv2 restriction is dependent on a specific pathway of entry (Schmitz et al., 2004).

The role of endocytosis and PM trafficking of CD4 in HIV infection has been reported previously, and cells that do not actively internalise CD4 are more infectable than cells that rapidly endocytose CD4 (Pelchen-Matthews et al., 1995). In keeping with this, Lv2 restricted virus infection is recovered by inhibiting endocytosis with hypertonic sucrose, by depletion of lipid rafts and by directing CD4 outside of lipid rafts, which supports the notion of an endocytic route of restricted virus internalisation.

CD4 is internalised by HeLa/CD4 cells in a clathrin dependent manner (Pelchen-Matthews et al., 1992; Pelchen-Matthews et al., 1995) and hypertonic media inhibit the formation of the clathrin triskelion on the PM (Heuser and Anderson, 1989). Cholesterol depletion has been suggested to inhibit clathrin dependent endocytosis as well as lipid raft dependent pathways in a Chinese hamster ovary cell line (Subtil et al., 1999), but there was no inhibition of Trf-FITC uptake in either HeLa/CD4 or U87/CD4/CXCR4 cells. Indirect evidence that Lv2 acts independently of clathrin is that restricted virus pseudotyped with a clathrin dependent VSV Env is rescued from Lv2 restriction (McKnight A, 2001; Schmitz et al., 2004; Sun et al., 2005). Lastly, the clathrin dominant negative mutant, Eps15 did not have an effect on restricted virus infection in restrictive cells confirming that a clathrin route of entry has little or no role in Lv2 restriction.

Recovery of HIV infection by treatment of target cells with pH neutralising reagents has been described previously and is dependent on the HIV isolate used (Fredericksen et al., 2002; Wei et al., 2005). However the pH-independence of Lv2 restriction shown by monensin and bafilomycin A1 treatment of restrictive cells suggest that acidified endosomes are not involved in Lv2 restriction. Furthermore, pseudotyping MCR with a pH-dependent VSV Env rescues Lv2 restricted virus infection (McKnight A, 2001; Schmitz et al., 2004), which further supports the model that Lv2 restriction occurs in a pH-independent compartment.

A more precise identification of the specific endocytic route(s) leading to Lv2 was determined using dominant negative mutants. Restricted virus infection is recovered when the Arf6 T27N dominant negative mutant is over-expressed in restrictive cells.

Additionally, infection is recovered when the dynamin K44A dominant negative mutant is over-expressed in HeLa/CD4 cells.

Double Arf6 and dynamin knock down experiments indicated that there was no additive effect on MCR suggesting that these mechanisms may be interconnected. The internalisation of M2 muscarinic acetylcholine receptor in HeLa cells is Arf6 dependent but dynamin independent (Delaney et al., 2002). In MDCK cells, Arf6 endocytic recycling requires the action of dynamin (Altschuler et al., 1999). Therefore, dynamin involvement in Arf6 trafficking may be cell-type dependent. But dynamin is involved in Lv2 restriction in HeLa/CD4 cells and is a part of the Arf6 component of restriction. HIV-2 MCR infection is not recovered more with the double Arf6/dynamin knockdowns compared to Arf6 or dynamin single knockdowns suggesting that these mechanisms are not mutually exclusive. HIV-1 89.6 on the other hand shows additive infection recovery when both Arf6 and dynamin are knocked down. This may be because, in this case, some restricted 89.6 is internalised by an Arf6-independent dynamin dependent pathway that eventually traffics 89.6 to the Arf6 early endosome. Indeed, the dominant negative clathrin Eps15 mutant recovers some restricted 89.6 but not MCR infection. The majority of 89.6 restricted virus may enter the Arf6 endosome by the Arf6/dynamin route, like MCR (fig 4.15). The transfer of proteins in HeLa cells between the Arf6 early endosome and the clathrin Rab5 early endosome via an intermediate compartment has been described (fig 4.15) (Delaney et al., 2002). This model is certainly favoured by the additive recovery of 89.6 infection in the double dynamin/Arf6 dominant negative knockout cells but not by the MCR infection recovery. To conclude, MCR and 89.6 may enter the restricting Arf6 compartment by different pathways.

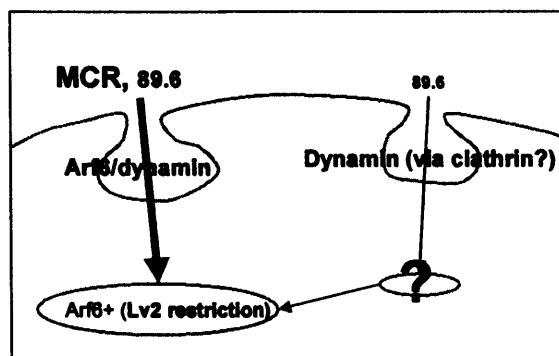


Figure 4.15. Dynamin and Arf6 mediated pathways of restricted HIV entry.

The viral *env* and *gag* genes act in concert to mediate Lv2 restriction (Schmitz et al., 2004), and mediate rescue of infection upon hypertonic sucrose treatment (Marchant et al., 2005). This suggests that the restricted virus Env targets the susceptible capsid cargo into the Lv2 compartment where the virus is restricted. The recovery of HIV infection in HeLa/CD4 cells with lysosomotropic agents has been reported (Fredericksen et al., 2002; Pelchen-Matthews et al., 1995) and like Lv2, the viral Env mediates the recovery of infection in this system (Wei et al., 2005). Interestingly, an MCN Env pseudotype of HIV-1 was rescued from an apparent restriction in HeLa/CD4 cells with sucrose treatment. This suggests that MCN Env directs HIV-1 into the Lv2 compartment (or Lv2-like) for restriction, suggesting that in the wild-type unrestricted MCN virus, the Gag and Env cooperate their activities to avoid Lv2 restriction. In other words, MCN Gag core has evolved to infect HeLa/CD4 cells via the Lv2 restriction route lending support to the theory that the viral *env* and *gag* must co-evolve to avoid restriction (Schmitz et al., 2004).

MβCD had little effect on the infectivity of the gene swap, MCNmcrenv and MCNmrgag, and only wild-type MCR infection was recovered on HeLa/CD4 cells with this treatment. This is in contrast to the recovery of MCR and MCNmcrenv infection in HeLa/CD4 cells with sucrose treatment. However, if the sucrose treatment and MβCD were acting at different stages along the restriction pathway this result may not be so surprising (fig 4.16). As reported previously, hypertonic media inhibit early events in endocytic uptake (Heuser and Anderson, 1989), HIV Env may direct the

virion into these sites hence the Env mediated recovery of restriction with hypertonic sucrose. Only wild-type MCR, with both MCR Env and Gag together, was rescued with M β CD treatment. This may suggest that by the time the virus reaches the M β CD compartment, Env has already performed its, restriction-targeting, role.

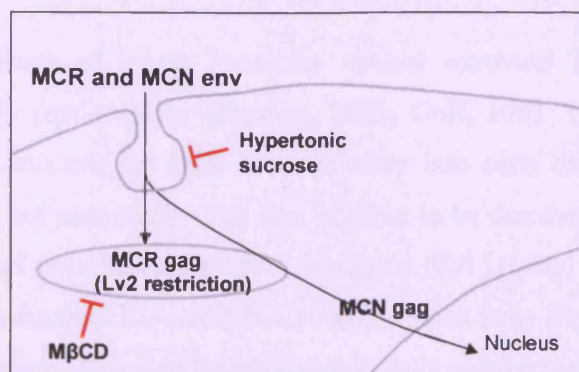


Figure 4.16. Methyl- β -cyclodextrin and sucrose may act on different stages of the restriction pathway.

In this chapter I show that both an (1) endocytic lipid raft pathway, elucidated with sucrose treatment and M β CD, and (2) a dynamin dependent Arf6 pathway, elucidated with dominant negative mutants, are involved in Lv2 restriction. However it has not been shown whether these two mechanisms of entry are the same or different pathways. Experiments where endocytosis was inhibited with M β CD or sucrose in cells expressing dominant negative endocytosis mutants proved too toxic for accurate analysis of results. Regardless, there are interesting similarities between the cholesterol requirements of the restriction and the Arf6 pathway; both the Arf6 pathway and Lv2 require lipid rafts and cholesterol (Hiroshima and Exton, 2005; Kirkham et al., 2005; Naslavsky et al., 2004). It is therefore possible that the Arf6 dominant negative characterisation of the restriction and the M β CD and hypertonic sucrose studies represent the same pathway of restriction.

Chapter 5

Lv2 is a saturable restriction

5.1 Introduction:

Numerous mechanisms of innate immunity against retroviral infection have been described previously (reviewed in (Bieniasz, 2003; Goff, 2003; Goff, 2004)). While some of these mechanisms act prior to virus entry into cells the recently described ‘restriction factors’ act post-entry. The first of these to be discovered and investigated in detail is the Friend virus susceptibility-1 restriction (Fv1) (Lilly, 1967). B-tropic and N-tropic Murine Leukaemia Viruses (MLV) readily infect cells from BALB/c and NIH Swiss mice, respectively, however BALB/c mouse cells restrict N-tropic MLV infection by approximately 1000-fold and NIH cells restrict B-tropic virus by 100-fold. The aptly named NB-tropic MLV can infect both cell types equally as well (Hartley et al., 1970). When the genetic locus of the Fv1 restriction was mapped it was discovered that the Fv1ⁿ and Fv1^b restriction phenotypes are alleles of the same *fv1* gene (Pincus et al., 1971).

Fv1 has been cloned and shares sequence similarity to a mouse endogenous retrovirus *gag* that is unrelated to MLV. Localised to mouse chromosome 4, Fv1 has its own retroviral major homology region (MHR) that it requires for restriction activity (Best et al., 1996). Regions at the N and C-terminal ends are also required to restrict N or B-tropic MLV infection (Bishop et al., 2001).

MLV can infect human cells if the MLV ecotropic Env is replaced with a pantropic VSV Env. These mixed MLV (VSV) pseudotypes were used to show that N-tropic MLV is restricted in human cells (Towers et al., 2000). The N-MLV restriction in human cells, termed ‘Ref1’, was later shown to also target EIAV (Hatzioannou et al., 2003). The pattern of Ref1 restriction, whereby N-tropic MLV but not B-tropic MLV is restricted, is also present in cells from a wide variety of mammals (Towers et al., 2000).

The target of Fv1 and Ref1 restrictions is the MLV capsid protein (CA). When residue 110 of the MLV CA is an acidic amino acid it is sufficient to confer B-tropism to MLV, whereas if a basic amino acid is substituted at this site the tropism switches to N-tropic (Kozak and Chakraborti, 1996). Ref1 was discovered using the N-tropic and B-tropic MLV Gag-Pol packaging constructs that differ at amino acid position 110 of capsid. Thus, it was apparent that, like Fv1, Ref1 was a CA directed restriction (Towers et al., 2000). However, unlike Fv1, Ref1 restriction was shown to occur before reverse transcription (Jolicoeur and Rassart, 1980; Towers et al., 2000; Yang et al., 1980). It has even been proposed that Fv1 requires the action of huTRIM 5 α to effect viral restriction when Fv1 is expressed in human cells (Keckesova et al., 2004).

Lv1 is a post-entry restriction to VSV-G pseudotyped HIV, SIV, and MLV in non-human primate cells (Cowan et al., 2002). However its activity in different primate cells varies slightly. Generally, Lv1 is more restrictive towards HIV-1 than SIV_{MAC} and MLV in Old World monkey cells (Hofmann et al., 1999). However, SIV_{MAC} is more restricted than HIV-1 in New World monkey cells (Hofmann et al., 1999). Examination of primary cells from various tissues suggests that the species and not the tissue of origin determine this restriction (Bieniasz, 2003; Hofmann et al., 1999). Lv1 in African green monkey cells has broader activity than Lv1 in other non-human primates or Ref1 in human cells because it can restrict N-tropic MLV, SIV_{mac}, HIV-1 and 2, and EIAV, (Besnier et al., 2002; Bieniasz, 2003; Cowan et al., 2002; Hatziiioannou et al., 2003). Lv1 is known to restrict HIV-1 in Rhesus macaque, and even rabbit and pig cells (Hatziiioannou et al., 2003; Hofmann et al., 1999). Like Ref1, Lv1 activity is directed against the CA of the incoming retrovirus and occurs pre-reverse transcription (Owens et al., 2004; Owens et al., 2003).

A characteristic of the Fv1, Ref1, and Lv1 restrictions is that they can be “abrogated” with saturating doses of ‘decoy’ virus-like particles (VLPs). The *in-vitro* saturation of restriction factors by pre-treatment with genome-defective particles was first described for Fv1 (Boone et al., 1990; Duran-Troise et al., 1977). Restriction abrogation experiments are performed by saturating cellular restriction factors with concentrated doses of VSV Env pseudotypes of MLV, HIV, and SIV Gag cores, as has been described for Ref1 and Lv1 [(Towers et al., 2002) and (Besnier et al., 2002)]

respectively], prior to challenge with sensitive virus resulting in loss of restriction (apparent increase in sensitive/reporter virus infection).

Lv2 is a restriction to HIV infection in human cells that is mediated by both the HIV Env and CA (Schmitz et al., 2004). In chapter 3 I introduced the model of Lv2 restriction of the molecular clones MCR and MCN in HeLa/CD4 cells and the prevalence of Lv2 among HIV-1 and HIV-2 viruses. Chapter 4 investigated how restricted viruses are directed into Lv2 restriction via an endocytic pathway (Arf6 and dynamin dependent) (Marchant et al., 2005), and reinforced the model that HIV Gag and Env act in concert to mediate Lv2 restriction.

In this chapter I set out to examine whether, similar to Ref1, Lv1 and Fv1, Lv2 is saturable. N and B-tropic MLV (MLV-N/B) Gag-Pol cores with MCR and MCN Env could saturate the Lv2 restriction factor and rescue restricted virus infection, however MLV-N/B with VSV Env had no effect. In contrast to Ref1, which restricts N but not B tropic MLV, I show that Lv2 can restrict both N and B tropic cores and that MLV-B has the most potent ability to absorb and abrogate Lv2 restriction factors. Thus in this way I was able to investigate the relationship between Ref1 and Lv2.

5.2 Results:

5.2.1 Lv2 is distinct from Ref1 in human cells.

Viruses that are susceptible to Ref1 restriction in human cells and Lv1 restriction in non-human primate cells are rescued by pre-absorption of the Ref1 restriction factors with saturating doses of decoy virus particles (Besnier et al., 2002). I sought to determine whether the pre-absorption of Ref1 by susceptible virus like particles (VLPs) on Lv2 restrictive cells could rescue restricted MCR. As a positive control, Ref1 saturating doses of VSV-G pseudotyped MLV-N and B VLPs with a zeomycin resistance transfer vector were used to rescue VSV Env pseudotypes of MLV-N Gag-Pol cores with an eGFP reporter vector from Ref1 restriction. Fig 5.1 shows that infection by MLV-B(VSV) pseudotypes are not enhanced by absorption with the decoy VLPs. The MLV-N(VSV) pseudotype infections were rescued in all of the cells tested

when absorption was carried-out with MLV-N(VSV) but not MLV-B(VSV) saturating VLPs (data not shown). MLV-N(VSV) titre increased by 7.5-fold in HeLa/CD4 cells. These results are consistent with those previously described for Ref1 restriction (Hatzioannou et al., 2003).

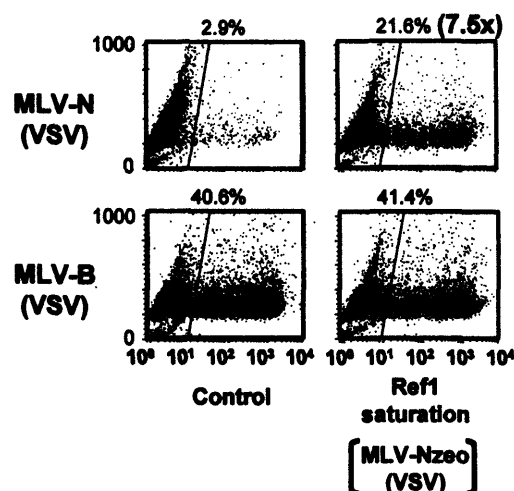


Figure 5.1. Ref1 can be saturated in HeLa/CD4 cells by pre-treatment with vesicular stomatitis virus envelope pseudotyped N-tropic MLV particles.

To confirm that Ref1 could be saturated with N-tropic MLV, stocks of MLV-N(VSV) were added to HeLa/CD4 cells at an MOI of 10. Four hr later, MLV-N(VSV) with a GFP reporter transgene was titrated onto the cells and GFP was detected 3 days later by flow cytometry. The results shown are representative of 2 independent experiments.

Fig 5.2 shows the titration of MLV-N(VSV) and MLV-B(VSV) on the Fv1-null cell line, *mus-dunni* tail fibroblasts (MDTF), a permissive cell, to determine the actual infectious units per ml of pseudotype stocks in the absence of an Fv1 restriction phenotype (Lander and Chattopadhyay, 1984). The infectious titre of MLV-N(VSV) and MLV-B(VSV) in MDTF cells are 3.5×10^5 and 4.0×10^5 infectious units per ml, respectively, as shown in fig 5.2. The titre of MLV-N(VSV) on HeLa/CD4 and U87/CD4/CXCR4 cells is 5.2×10^3 and 20 infectious units per ml respectively, indicating that both Lv2 restrictive and permissive cells are Ref1 positive.

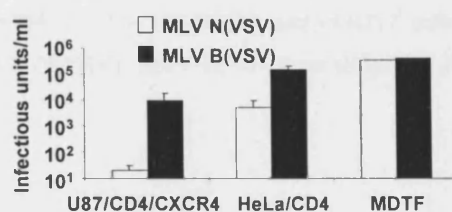


Figure 5.2. Permissive U87/CD4/CXCR4 cells have more Ref1 activity than Lv2 restricted HeLa/CD4 cells.

MLV-N(VSV) and MLV-B(VSV) are equivalent titre in Fv1 null MDTF cells. MLV-N(VSV) and MLV-B(VSV) vector pseudotypes were titrated in Ref1 positive U87/CD4/CXCR4, HeLa/CD4, and on Fv1 null MDTF cells. Infectious units were determined by flow cytometry. Results shown are representative of 3 independent experiments. Error bars represent the SDM.

To determine whether MLV-N(VSV) vector pseudotype could saturate Lv2 I performed a saturation experiment. Lv2 sensitive MCR was titrated on restrictive HeLa/CD4, GHOST, and permissive U87/CD4/CXCR4 cells (McKnight A, 2001; Schmitz et al., 2004), that had been pretreated with Ref1-saturating doses of MLV-N(VSV) (fig 5.3). The titre of MCR increased in the MLV-N(VSV) treated HeLa/CD4, GHOST, and U87/CD4/CXCR4 cells by factors of 5.0, 2.0, and 2.5-fold, respectively, over the mock transfection control. Therefore, pre-treatment of all cells with MLV-N(VSV), regardless of whether they were Lv2 susceptible or not, resulted in increases in MCR titres. Thus there was no abrogation of Lv2 restriction with MLV-N or B(VSV).

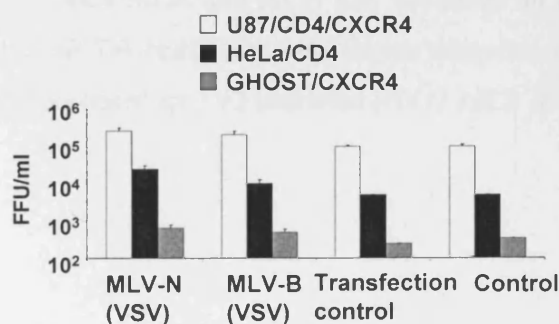


Figure 5.3. The infectious titre of Lv2 restricted MCR is not affected by saturation of Ref1 with vesicular stomatitis virus envelope pseudotypes of N-tropic MLV.

MCR was titrated on U87/CD4/CXCR4, HeLa/CD4, and GHOST cells that had been pre-treated with Ref1-saturating doses of MLV-N(VSV). MLV-B(VSV) was added as a Ref1 negative control.

5.2.2 MCR envelope confers Lv2-like restrictions to N-tropic, B-tropic, and Moloney MLV virus-like particles.

It has been reported that a VSV Env rescues MCR in restrictive cell types. Furthermore MCR Env will confer restriction to resistant HIV-2 MCN and HIV-1 NL4.3 (Schmitz et al., 2004). The effect that MCR, MCN, and VSV Envs have on infection of MLV-N, B, and Mo Gag-Pol cores in HeLa/CD4, H399 and U87/CD4/CXCR4 cells was determined. The results of the flow cytometric analysis are illustrated in figs 5.4a, c, and e. The fold restriction (U87/CD4/CXCR4 divided by either the restricted HeLa/CD4 titre or permissive H399 titre) is shown in fig 5.4b, d, f.

The titres of VSV-G pseudotyped MLV-N, B and Mo Gag-Pol cores are equivalent on U87/CD4/CXCR4, HeLa/CD4, and H399 cells. The 55.0-fold lower titre of MLV-N(VSV) compared to MLV-B(VSV) is expected, due to Ref1. However when the MCR Env was used to pseudotype MLV cores this conferred fold restrictions in HeLa/CD4 cells of 35.7, 57.0-fold and 65.0-fold to MLV-B, MLV-N and MLV-Mo cores, respectively (figs 5.4b, d, and f). Interestingly, the 'unrestricted' MCN Env conferred a 14.9-fold restriction to the MLV-B core and a small but noteworthy 8.3-fold restriction to the MLV-Mo core. No restriction by MCN Env to MLV-N was detected in HeLa/CD4 cells. Where MCR and MCN Env conferred an Lv2-like restriction to the MLV cores in HeLa/CD4 cells there was almost complete recovery of restriction by H399 cells, as demonstrated for Lv2 restricted HIV-2 MCR in chapter 4.

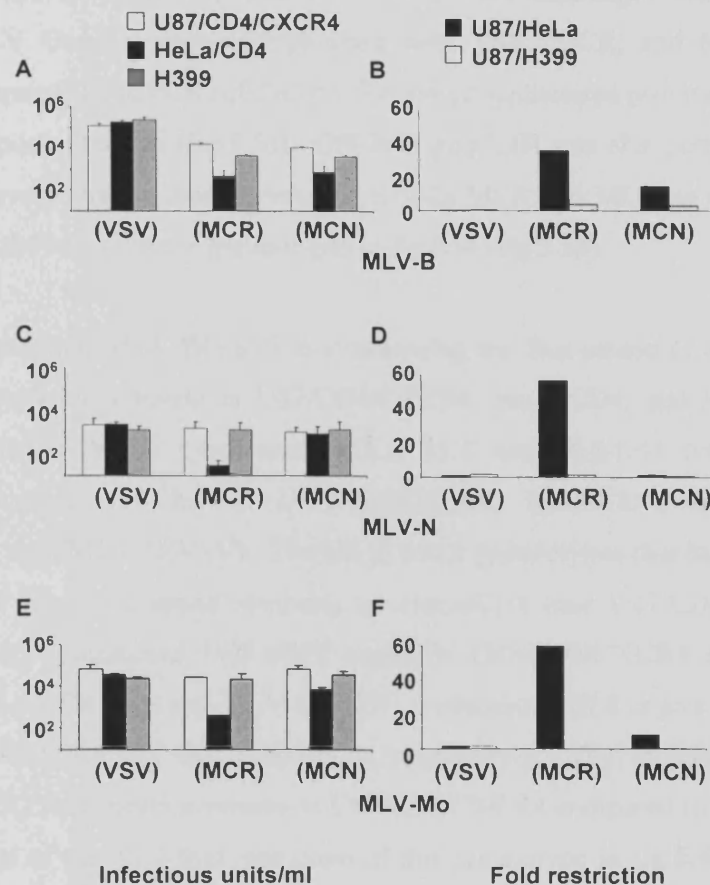


Figure 5.4. MCR envelope confers Lv2 restriction to N-tropic, B-tropic and Moloney MLV Gag-Pol cores.

(A) MLV-B (C) MLV-N (E) and MLV-Mo pseudotypes with VSV, MCR, and MCN Envs were titrated on U87/CD4/CXCR4, HeLa/CD4, and H399 cells. Three days later, eGFP positive cells were determined by flow cytometry and the infectious units per ml were calculated. Fold restriction of the (B) MLV-B (D) MLV-N and (F) MLV-Mo pseudotypes were calculated by dividing the infectious titre on U87/CD4/CXCR4 by the titre from HeLa/CD4 or H399.

5.2.3 Restriction of MLV occurs pre-reverse transcription whereas restriction of HIV-2 occurs post-reverse transcription.

Lv2 restriction occurs post-reverse transcription but Ref1 occurs pre-reverse transcription (McKnight A, 2001; Towers et al., 2002). I therefore wanted to determine the point at which MLV-N and B are restricted in these human cells.

U87/CD4/CXCR4, HeLa/CD4, and H399 cells were challenged with MLV-N and MLV-B MLV Gag-Pol cores pseudotyped with VSV, MCR, and MCN Envs and analysed by quantitative PCR (qPCR) (to first strand synthesised proviral DNA) 10 min, 3 and 18 hr post-infection (fig 5.5a). QPCR of *gag*-LTR was also performed to detect full-length reverse transcribed provirus of HIV-2s MCR and MCN as a comparison to the qPCR of the MLV vector pseudotypes in fig 5.5a (fig 5.5b).

Fig 5.5a shows that MLV-B(VSV) is synthesising the first strand of the MLV vector genome to equivalent levels in U87/CD4/CXCR4, HeLa/CD4, and H399 cells. As expected, MLV-N(VSV) synthesised 111.2, 11.0 and 76.5-fold fewer first strand reverse transcripts (18 hr) in U87/CD4/CXCR4, HeLa/CD4, and H399 cells, respectively, than MLV-B(VSV). The MLV vector pseudotypes that have an MCR Env demonstrated less first strand synthesis on HeLa/CD4 than U87/CD4/CXCR4 cells. MLV-N(MCR) synthesised 14.0 eGFP copies in U87/CD4/CXCR4 compared to 2.0 copies in HeLa/CD4 cells and MLV-B(MCR) synthesised 307.0 copies compared to 6.0 in U87/CD4/CXCR4 and HeLa/CD4 cells, respectively. The 48.0-fold difference in MLV-B(MCR) first strand synthesis in U87/CD4/CXCR4 compared to HeLa/CD4 cells is reminiscent of the 35.7-fold restriction of this pseudotype in fig 5.4b. The 7.0-fold drop in first strand copies of MLV-N(MCR) from 14.0 to 2.0 copies in U87/CD4/CXCR4 and HeLa/CD4 cells, respectively, is considerably less than the 57.0-fold restriction of this pseudotype in fig 5.4d. This number of qPCR copies is however at the lower limit of detection and therefore less accurate. The MLV-N(MCN) pseudotype was also at the lower limit of qPCR detection and the number of copies in U87/CD4/CXCR4 and HeLa/CD4 cells was an equivalent 9.0 and 7.0 copies but this is reminiscent of the lack of restriction of MLV-N(MCN) in U87/CD4/CXCR4 and HeLa/CD4 cells in fig 5.4d. Since the synthesis of the first strand qPCR in fig 5.5a matches the drop in titre of these vector pseudotypes during restriction in fig 5.4 the restriction of MCR and MCN pseudotyped MLV cores is therefore occurring pre-reverse transcription.

The above data is in contrast to that seen for Lv2 restriction. The restriction of HIV-2 MCR in HeLa/CD4 cells occurs post-reverse transcription as shown in fig 5.5b, and described previously (McKnight A, 2001; Schmitz et al., 2004). At 18 hr post-infection, HIV-2s MCR and MCN demonstrate 20.0 and 40.0 copies, respectively, of

full-length *gag*-LTR provirus. This 2.0-fold difference does not account for the 40.0-fold restriction between MCR and MCN in HeLa/CD4 cells in fig 3.8 of chapter 3.

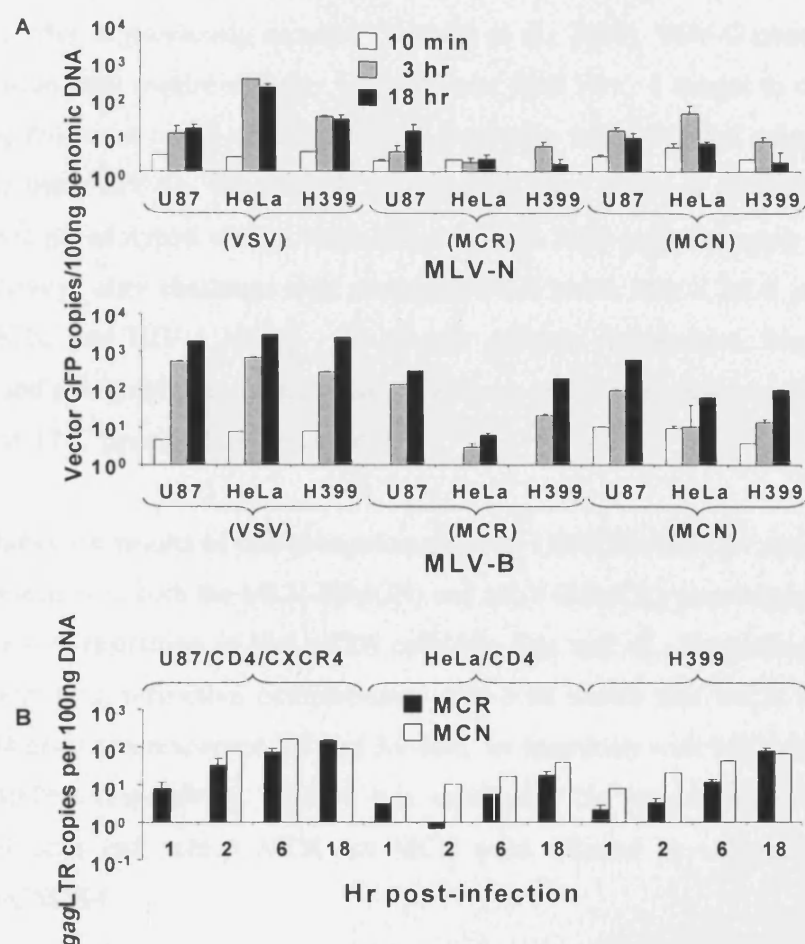


Figure 5.5. Lv2 restricted MLV is blocked pre-reverse transcription whereas Lv2 restriction of MCR occurs post-reverse transcription.

(A) qPCR of first-strand cDNA synthesis by MLV-N and B Gag-Pol pseudotypes. Infections were synchronised on ice then incubated at 37°C for 10 min, 3 hr, and 18 hr time points and eGFP qPCR was performed on the cellular DNA. Envs used to pseudotype MLV-N and B Gag-Pol are indicated in brackets: (MCR), (MCN) and (VSV). (B) QPCR for full-length reverse transcripts of MCR and MCN 1, 2, 6, 18 hr post-infection in U87/CD4/CXCR4, HeLa/CD4, and H399 cells. The results shown are representative of 4 independent experiments in (A) and 3 independent experiments in (B). Error bars represent the standard error of the mean.

5.2.4 B-tropic MLV pseudotype with an HIV-2 envelope can abrogate Lv2 and rescue restricted HIV-2 MCR and HIV-1 89.6 infection.

As shown in fig 5.3, MLV-N or B(VSV) vector pseudotypes do not rescue Lv2 restriction. But as previously reported (Schmitz et al., 2004), VSV-G overcomes Lv2 and saturation may require delivery by a different viral Env. I sought to determine if MLV Gag-Pol cores could saturate-out Lv2 restriction when targeted using an HIV-2 Env rather than VSV-G. Since MCR Env was the most potent at restricting MLV-N and B these pseudotyped viruses were added at high MOI (approximately 10^4 pg RT ELISA activity) after challenge with sensitive HIV-2 MCR, HIV-1 89.6, and resistant HIV-2 MCN, and HIV-1 NL4.3. To prevent receptor interference, binding of the indicator and abrogating/saturating viruses was done on ice and uptake into the cell was triggered at 37°C (materials and methods).

Fig 5.6 shows the results of this abrogation assay in U87/CD4/CXCR4 and HeLa/CD4 cells. Interestingly, both the MLV-B(MCN) and MLV-B(MCR) pseudotypes were able to saturate Lv2 restriction in HeLa/CD4 cells (fig 5.6c and d). Suggesting that MCN Env delivers to a restrictive compartment. Fig 5.6d shows that MCR infection on HeLa/CD4 cells was recovered 3.5 and 3.0-fold, by saturation with MLV-B(MCR) and MLV-B(MCN), respectively. MCN was unaffected by any of the treatments in HeLa/CD4 cells and neither MCR nor MCN were affected by abrogating doses in U87/CD4/CXCR4.

Infection by HIV-1 89.6, the Lv2 restricted HIV-1, in HeLa/CD4 cells increased from 18 to 120 FFU upon abrogation with the MLV-B(MCR) pseudotype and 72.5 FFU with MLV-B(MCN) (fig 5.6c). This corresponds to recovery of 89.6 infection of 6.7-fold and 4.0-fold for the MLV-B(MCR) and MLV-B(MCN) pseudotypes, respectively (fig 5.6d). HIV-1 NL4.3 infection in U87/CD4/CXCR4 and HeLa/CD4 cells was relatively unaffected by these treatments.

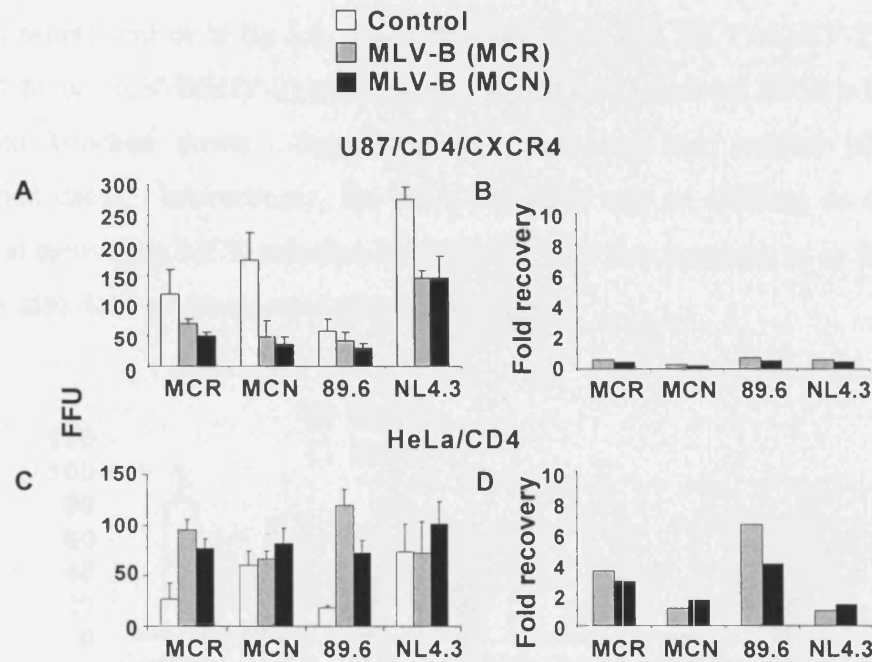


Figure 5.6. B-tropic MLV pseudotypes with HIV-2 envelopes abrogate Lv2 restriction and recover restricted HIV-1 and HIV-2 virus infection.

Fixed doses of HIV-2s MCR, MCN and HIV-1s 89.6 and NL4.3 were plated on (A) U87/CD4/CXCR4, and (C) HeLa/CD4 cells on ice. Neat pseudotype supernatant was plated over the HIV-2 and HIV-1 challenge virus on ice for a further 30 min, incubated at 37°C, then fixed and stained for HIV-2 infection 3 days later. (B,D) The FFU from the MLV(HIV-2) saturations were divided by the FFU from the control to obtain fold recovery. Results shown are representative of 12 independent experiments. Error bars represent the SDM.

5.2.5 Ref1 (human tripartite motif protein 5 α) reduces the potency of HIV-2 envelope pseudotypes of N-tropic MLV to saturate out the factor responsible for Lv2 restriction.

Figs 5.6 and 5.7a show that MLV-B(HIV-2) pseudotyped particles can saturate Lv2 restriction and partially rescue some restricted HIV-1 and 2 infection. Since MLV-N(VSV) is known to be Ref1 restricted by huTRIM 5 α , I wanted to determine if the potency of MLV-N(HIV-2) particles to saturate Lv2 restriction could be improved by knocking down TRIM 5 α . I noted that MLV-N pseudotypes with HIV-2 Envs were unable to saturate Lv2 and recover MCR infection as in the experiment in fig 5.7a. HeLa/CD4 cells were treated with TRIM 5 α RNAi before carrying-out an Lv2

saturation experiment as in fig 5.6. Fig 5.7b and c show that MLV-N(HIV-2) particles are as potent as MLV-B(HIV-2) particles at rescuing Lv2 restricted MCR when TRIM 5 α is first knocked down. Suggesting that Ref1 can also restrict HIV-2 Env pseudotyped cores. Interestingly, the MLV-N(MCN) was as efficient as the MLV-N(MCR) at recovering MCR infection by 5.3-fold. This also suggests, as in fig 5.6, that MCN Env also delivers into a restrictive compartment.

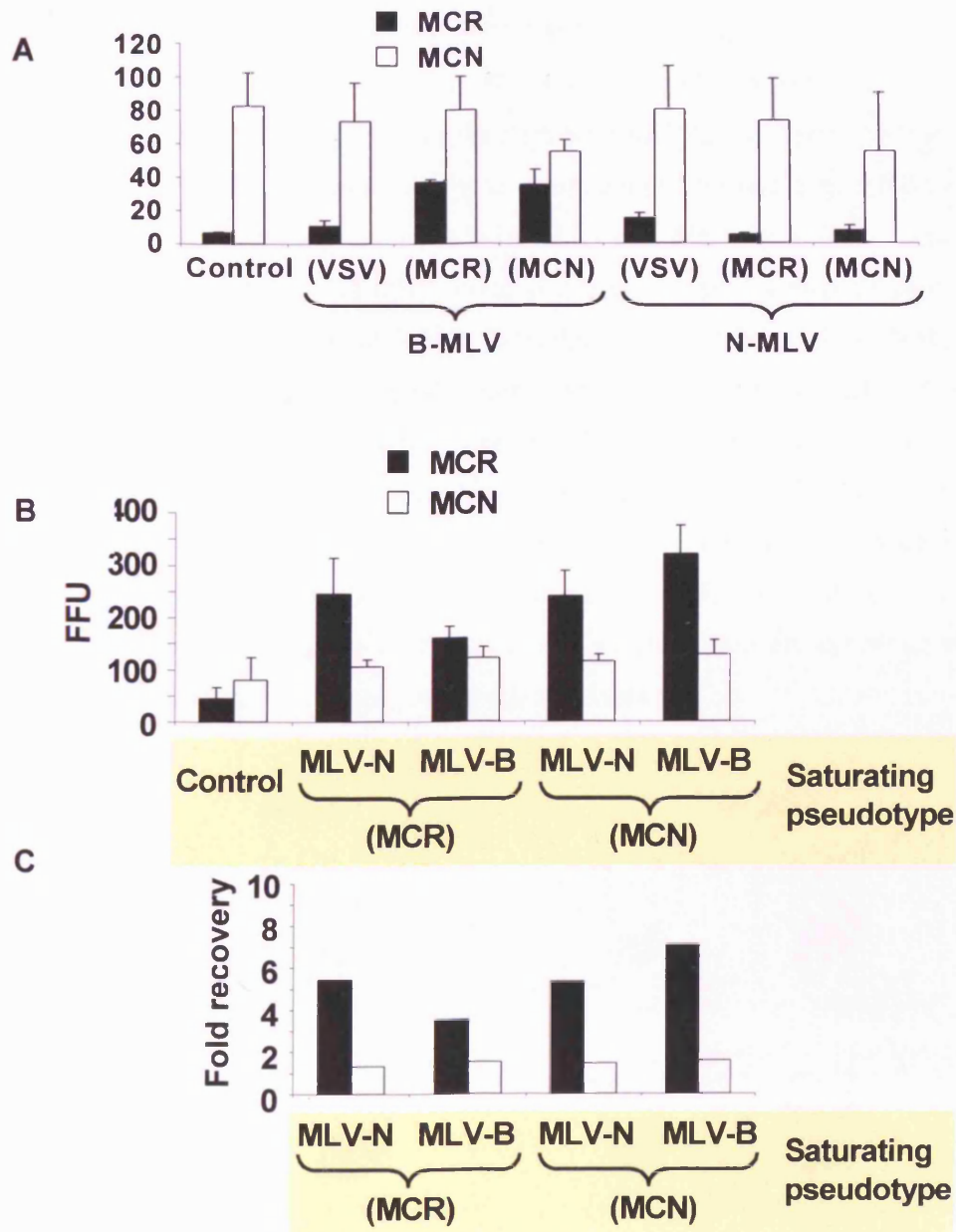
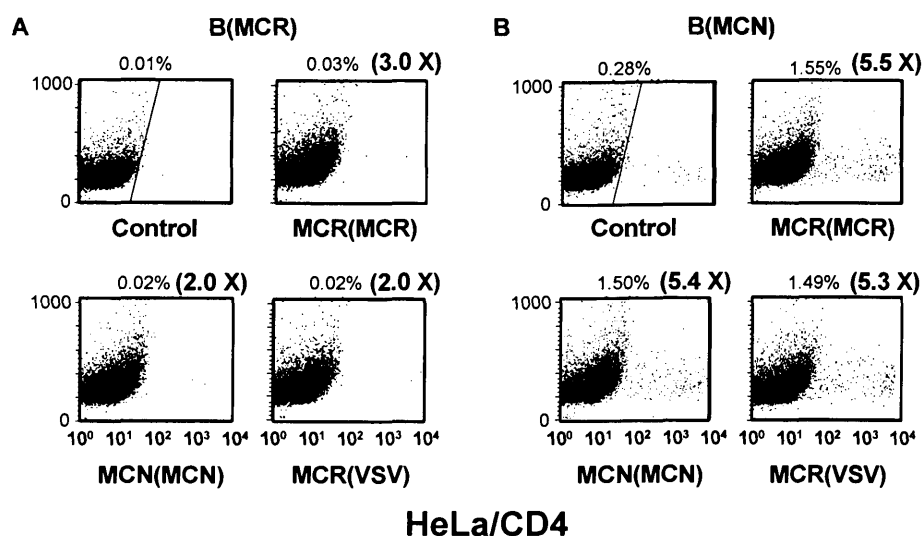


Figure 5.7. N-tropic MLV Gag-Pol core can abrogate Lv2 restriction if Ref1/TRIM 5 α is 'knocked down' prior to challenge.

HeLa/CD4 cells were (B) treated with RNAi oligomers directed against TRIM 5 α or (A) without and abrogation experiments were performed as in fig 5.6. (C) The fold recovery (shown below) was obtained by dividing the FFU from the MLV(HIV-2) pseudotype treated infection by the FFU from the control infection in (B). Results are representative of 3 independent experiments. Error bars represent the SDM.

5.2.6 Infection by HIV-2 envelope pseudotypes of B-tropic MLV can be enhanced in Lv2 restrictive HeLa/CD4 and permissive H399 cells with abrogating doses of HIV-2: evidence for other restriction pathways.

HIV-2 Env pseudotypes of MLV-N and -B can saturate Lv2 and partially recover HIV-2 and HIV-1 infection on HeLa/CD4 cells (figs 5.6 and 5.7). To determine whether the same pattern of recovery would occur with saturation of Lv2 with high MOI of HIV-2 virus followed by challenge with MLV-B(MCR) and MLV-B(MCN). Dilutions of MLV-B(MCR) and MLV-B(MCN) vector pseudotypes were bound to cells on ice together with high MOI doses of HIV-2. In HeLa/CD4, the titre of MLV-B(MCR) was not rescued to a large extent by pre-treatment with MCN, MCR, and MCR(VSV) (fig 5.8a). Infectivity of MLV-B(MCN) on HeLa/CD4 was enhanced more than 5-fold by pre-treatment with MCN, MCR, and MCR(VSV) respectively (fig 5.8b). Rescue of both MLV-B(MCR) and MLV-B(MCN) on H399 cells was more pronounced with 7 to 10-fold recovery with MCN, MCR, and MCR(VSV) generally seen, in figs 5.8c and d. The results suggest alternative mechanisms of Lv2 restriction are operating and that saturation experiments need to be interpreted with caution.



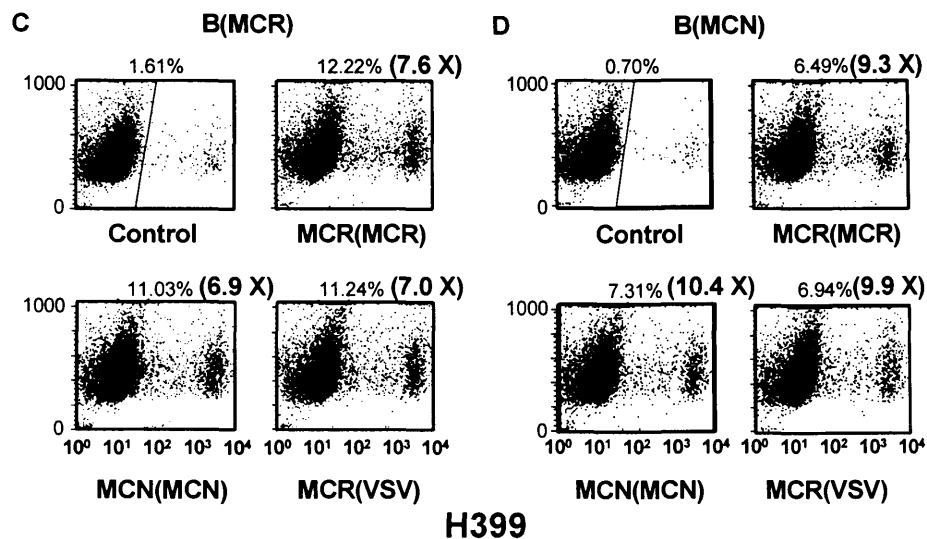


Figure 5.8. Reciprocal abrogation: infection by HIV-2 envelope pseudotypes of B-tropic MLV is rescued on both Lv2 restrictive and permissive cell types upon abrogation with HIV-2.

RT ELISA standardised inputs of MLV pseudotypes were added to (A, B) HeLa/CD4 and (C, D) H399 cells on ice. Neat supernatant (approx 10^4 pg RT U) of MCR, MCN, and MCR(VSV) were used to pre-treat cells. Cells were harvested two days later and eGFP positive cells were determined by flow cytometry. (# x) denotes the fold recovery over control. The results shown are representative of 3 independent experiments.

5.3 Discussion:

The results shown here demonstrate that MLV-N and -B pseudotyped with VSV-G are unable to saturate Lv2, resulting in no recovery of HIV-2 MCR infection. Substitution with either an MCR or MCN Env results in abrogation of Lv2 and recovery of restricted HIV-1 89.6 and HIV-2 MCR. These results support the model that Env plays a significant role in the susceptibility of virus to restriction factors. Thus MLV-B Gag-Pol particles with either HIV-2 MCR or MCN Envs can saturate-out Lv2 restriction and rescue restricted HIV-1 and HIV-2 virus infection. Initial experiments indicated, however, that MLV-N could not saturate in an Lv2 manner. Later experiments demonstrated that MLV-N could saturate Lv2 if the TRIM 5 α activity of Ref1 was first

knocked down. Thus Lv2 is independent from Ref1 restriction, Lv2 is able to recognise MLV-N, B, and Mo cores and is dependent on the Env route of entry.

Lv2 is an independent restriction directed by the viral env-receptor interaction. VSV Env overcomes Lv2 (Schmitz et al., 2004), indicating localisation of the Lv2 restriction factor in a distinct compartment.

This chapter further supports the model that Ref1 and Lv2 are distinct factors in human cells because; MLV-N Gag-Pol VLPs have lower titre on human cells than MLV-B VLPs, regardless of their Env, which is probably due to Ref1 restriction, however all MLV Gag-Pols tested became susceptible to Lv2 restriction in HeLa/CD4 cells when pseudotyped with MCR Env; MLV-B(MCR) is restricted by Lv2 but not by Ref1; the discreet compartmentalisation requires that Lv2 is only saturable by MLV Gag-Pol cores with HIV-2 Envs and finally, MLV-N(HIV-2) can saturate Lv2 restriction if TRIM 5 α is knocked down. This suggests that Lv2 and Ref1 compete for incoming retroviral cores and that Ref1 may occur before Lv2 in the host cell. Alternatively, Ref1 could be dominant over Lv2 activity when competing for incoming retroviral capsids.

Lv2, like ref1, can be abrogated by saturation with MLV Gag ‘decoy’ VLPs. However, unlike Ref1 restriction, both MLV-B and MLV-N cores are able to saturate Lv2 restriction. Surprisingly, MLV-B VLPs with both restricted MCR and unrestricted MCN Envs could saturate Lv2 restriction and recover HIV-1 89.6 and HIV-2 MCR infection on HeLa/CD4 cells. Suggesting that MCN Env can deliver to both a restrictive and non-restrictive pathway.

A number of experiments presented in this chapter suggest that saturation experiments need to be interpreted with caution. 1) The MLV-N and Mo Gag may be targeted by additional restriction factors other than those specific to Lv2 thus decreasing their potency to knockout Lv2 restriction and rescue MCR infection; TRIM 5 α knockdown by RNAi improved the potency of MLV-N to saturate Lv2. 2) The ability of one species of virus core to saturate a restriction and rescue infectivity of another species of virus core cannot always be reciprocated

In the 'reciprocal abrogation' experiments MLV-B(HIV-2) vectors were rescued with saturating doses of HIV-2. Recovery of MLV-B(HIV-2) was in a non-Lv2 manner; there was recovery of MLV-B(HIV-2) in Lv2 permissive cells. Therefore, Lv2 permissive cells may bypass Lv2 restrictive compartments but direct viruses into previously undescribed restrictive routes.

This chapter has shown that, like Ref1, Lv2 is a saturable restriction factor but, unlike Ref1, Lv2 is compartment specific. Since Lv2 is saturable it suggests that a specific protein is responsible for the restriction of HIV-1 and HIV-2 in human cells. Chapter 6 describes the identification and characterisation of TRIM proteins that mediate Lv2 and these TRIMs can be bypassed with a VSV Env, further confirming that Lv2 and Ref1 are distinct restrictions.

Chapter 6

Characterisation of the tripartite motif proteins responsible for Lv2 restriction

6.1 Introduction:

In chapter 4 I partially characterised a specific compartment/pathway that leads to Lv2 restriction, and in chapter 5 I demonstrated the saturable nature of Lv2. This chapter characterises the restriction more specifically; identifying the cellular TRIM proteins involved in restriction of HIV-1 89.6 and HIV-2 MCR, and their cell-type specific expression.

TRIMs have been implicated as antiviral molecules (Nisole et al., 2005; Reymond et al., 2001). Indeed, TRIM 5 α was cloned from humans and rhesus macaques and identified as the Ref1 and Lv1 restriction factors, respectively (Keckesova et al., 2004; Perron et al., 2004; Stremlau et al., 2004; Yap et al., 2004). TRIM 5 α (Keckesova et al., 2004; Perron et al., 2004; Stremlau et al., 2004; Yap et al., 2004) and TRIM 1 (Yap et al., 2004) are responsible for the Ref1 and Lv1 restrictions and as outlined in the introduction to the last chapter, Ref1 is the human model for resistance to infection of MLV-N and EIAV (Hatzioannou et al., 2003; Towers et al., 2000). Lv1 restriction is the model for resistance of Rhesus macaque cells to HIV-1 and SIV_{AGM} infection (Hatzioannou et al., 2003), and African green monkey cells to HIV-1 and SIV_{MAC} infection (Besnier et al., 2002; Cowan et al., 2002; Hatzioannou et al., 2003). Therefore, TRIM 5 α and TRIM 1 are thought to be responsible for the cross-species lentivirus restrictions in humans and simians (Hatzioannou et al., 2004; Keckesova et al., 2004; Stremlau et al., 2004; Yap et al., 2004).

Because TRIM 1 and TRIM 5 α from humans and African green monkeys were shown to have Ref1 activity and TRIM 19 has been shown to have anti-viral activity against VSV, it was suggested that the TRIM protein family has very broad anti-viral activity (Yap et al., 2004).

TRIMs have also been associated with restrictions of viruses other than retroviruses. The promyelocytic leukaemia protein (PML), TRIM 19, has been demonstrated to have suppressive activity against numerous viruses including influenza and VSV (Chelbi-Alix et al., 1998), however this activity was shown to be at the stage of virus expression and not entry (Chelbi-Alix et al., 1998). PML has also been shown to inhibit Arenavirus infection in mice and may have antiviral effects on Herpes Simplex virus in human cells (Bonilla et al., 2002; Chee et al., 2003). With regards to HIV, there has been one report of PML associating with the incoming HIV-1 PIC in human cells and inhibiting HIV integration (Turelli et al., 2001). In this study, an enhancement of HIV-1 infection was shown upon treatment of cells with arsenic, however this treatment has been shown to suppress Ref1 restriction and recover HIV-1 infection in human cells by a PML independent mechanism (Berthoux et al., 2003; Keckesova et al., 2004; Sayah and Luban, 2004; Turelli et al., 2001).

Since the discovery of TRIM 5 α there has been great interest in the regions of this protein that are responsible for retroviral restriction. The SPRY (B30.2) domain was cited as the principal TRIM 5 domain responsible for antiviral activity (Stremlau et al., 2004; Stremlau et al., 2005). In particular, residues 320 to 345 have been proposed to be responsible for the species-specific restriction activity of TRIM 5 α (Nakayama et al., 2005; Perez-Caballero et al., 2005; Sawyer et al., 2005; Yap et al., 2005). More specifically, a proline to arginine alteration at aa position 332 in the SPRY domain has been shown to confer anti-HIV-1 activity to human TRIM 5 α , which does not normally have activity against this virus (Yap et al., 2005). There are four splice variants of TRIM5: α , δ , γ and ϵ [reviewed in (Nisole et al., 2005)]. However, of these, only the α splice variant can effect Ref1 and Lv1 activity (Keckesova et al., 2004; Stremlau et al., 2004; Yap et al., 2004). In fact, antiviral activity of the SPRY domain was first implicated by comparing the activities of TRIM 5 α with TRIM 5 γ , which is identical to TRIM 5 α except that TRIM 5 γ lacks most of the SPRY domain due to alternative splicing (Stremlau et al., 2004). In addition to the SPRY domain, MLV-N restriction by TRIM 5 α requires regions in the coiled-coil domain (Perez-Caballero et al., 2005; Yap et al., 2005).

Because Lv2 is a saturable restriction factor (shown in chapter 5), and CA dependent as reported previously (Schmitz et al., 2004), I investigated whether the TRIM proteins are

responsible for Lv2 and if one of them is TRIM 5 α , like for Ref1. Since TRIM 1 and TRIM 5 α both have SPRY domains and TRIM 5 α restricts via this domain the main criteria for choosing candidate Lv2 TRIMs, from the 69 TRIM genes identified, was the presence of a SPRY domain [reviewed in (Nisole et al., 2005)].

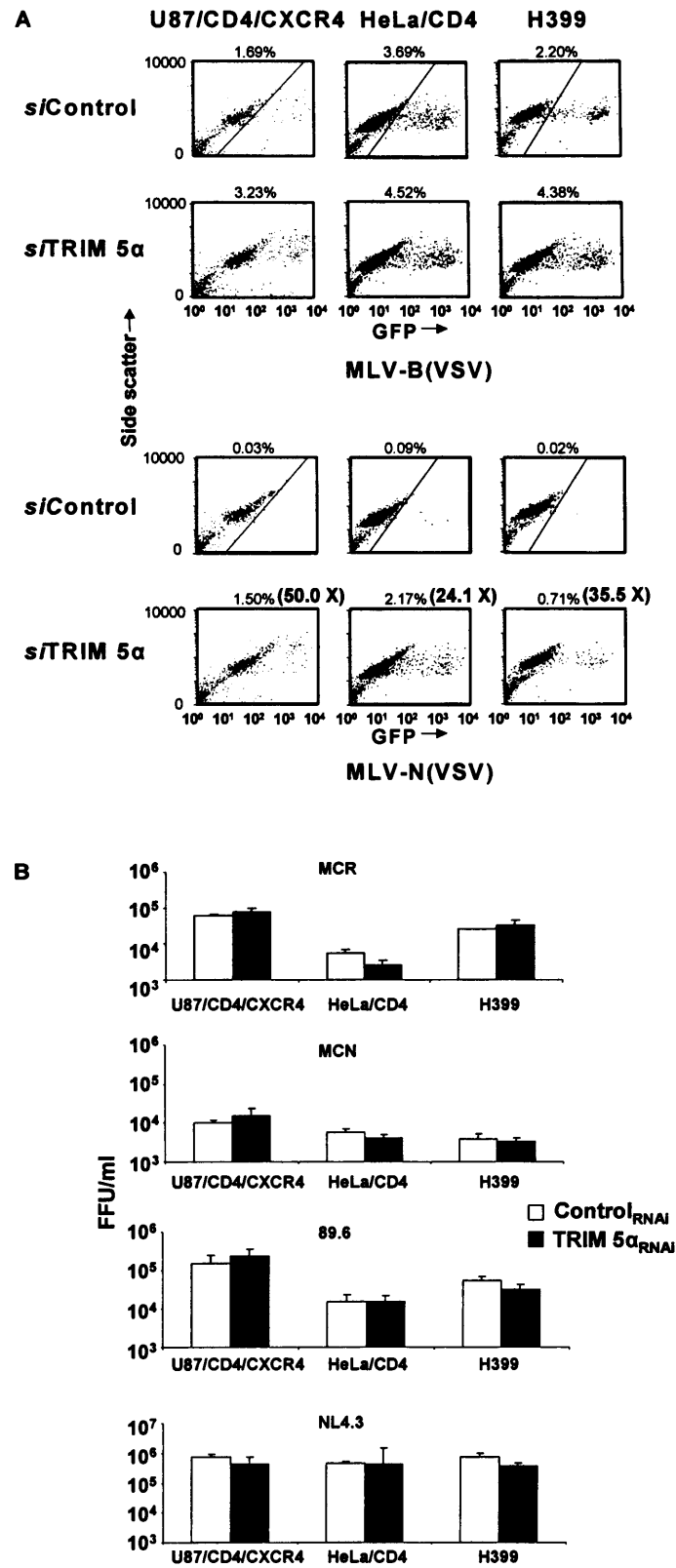
6.2 Results:

6.2.1 Tripartite motif protein 5 α is not responsible for Lv2 restriction in U87/CD4/CXCR4 and HeLa/CD4 cells.

I used RNAi to determine whether TRIM 5 α is responsible for Lv2 restriction. MLV cores with VSV Env could not saturate Lv2 restriction [chapter 5, (Schmitz et al., 2004)]. However, MCR pseudotyped with a VSV Env is not restricted. Thus it is still possible that TRIMs in the appropriate compartment could act as Lv2.

I first confirmed that the siRNA designed could knockdown TRIM 5 α activity in the cells used in this study. U87/CD4/CXCR4, HeLa/CD4, and H399 cells were transfected with TRIM 5 α and control RNAi oligomers, then infected with MLV-N(VSV) and MLV-B(VSV) VLPs (fig 6.1a). The titre of MLV-B(VSV) on the TRIM 5 α knockdown cells is not altered when compared to the control cells. MLV-N(VSV) titre increased by 50.0, 24.1, and 35.5-fold on the TRIM 5 α knockdown U87/CD4/CXCR4, HeLa/CD4, and H399 cells, respectively, over the control cells (fig 6.1a). These observations are consistent with those published previously (Keckesova et al., 2004).

The TRIM 5 α RNAi knockdown cells were titrated with restricted MCR, HIV-1 89.6 and unrestricted MCN and HIV-1 NL4.3 (fig 6.1b). There was little or no effect on the titre of HIV-2 MCR (5.3×10^4 FFU/ml vs 2.6×10^4 FFU/ml) or HIV-1 89.6 (1.5×10^4 FFU/ml vs 1.5×10^4 FFU/ml) on HeLa/CD4 cells, demonstrating no rescue of Lv2 restriction. 89.6 titre on control U87/CD4/CXCR4 cells was 1.53×10^5 FFU/ml increasing to 2.39×10^5 FFU/ml on the TRIM 5 α knockdown, increasing 89.6 restriction from 10.2 to 15.4-fold upon TRIM 5 α knockdown, however this does not appear to be an Lv2 specific result. Thus knockdown of TRIM 5 α /Ref1 activity did not alleviate Lv2 restriction.



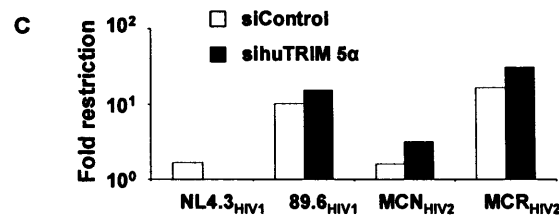


Figure 6.1. Knockdown of the tripartite motif protein 5α/Ref1 restriction factor has no effect on Lv2 in restrictive HeLa/CD4 cells.

(A) TRIM 5α RNAi knockdown cells rescue Ref1 restriction of MLV-N. Cells knocked down for TRIM 5α expression with RNAi were infected with MLV-N(VSV) and MLV-B(VSV). EGFP positive cells were determined by FACS and eGFP positive cells per ml were calculated. (B) RNAi directed against TRIM 5α has no effect on Lv2 restriction. Cells transfected with TRIM 5α RNAi or control RNAi oligomers were challenged with HIV-1s 89.6, NL4.3, and HIV-2s MCR, and MCN. (C) Fold restriction. The titre from permissive U87/CD4/CXCR4 was divided by the titre from restrictive HeLa/CD4 cells for both the control and the TRIM 5α RNAi experiments. Shown are the results of one experiment. Results in this fig are representative of 3 independent experiments. Error bars represent the SDM.

6.2.2 Members of the tripartite motif family of proteins can be knocked down specifically.

RNAi knockdown of TRIM 5α had no effect on Lv2 restriction of HIV-1 89.6 or HIV-2 MCR. However, because of the similarity between the restriction of HIV-1 by rhTRIM 5α in macaque cells and Lv2 restriction of HIV (Lv2 is saturable), other members of the TRIM family were screened for Lv2 activity.

I next investigated other SPRY domain containing TRIMs for anti-HIV Lv2 activity. TRIMs 1, 6, 18, and 34 were first investigated. There have been reports of non-specific silencing of non-target mRNA and protein expression in RNAi experiments (Doench et al., 2003; Jackson et al., 2003; Persengiev et al., 2004; Scacheri et al., 2004). I first determined the specificity of the various siRNAs used in the RNAi knockdown experiments by monitoring expression of TRIM-eGFP and endogenous TRIM mRNA production. RNAi oligomers directed against TRIMs 1, 6, 18, and 34 were designed, HeLa/CD4 cells were transfected with the RNAi oligomers, transfected with TRIM 1, 6 and 18-GFP plasmid constructs and fig 6.2a shows approximately 90% knockdown of

eGFP expression. TRIM RNAi did not inhibit expression of heterologous TRIM-eGFP constructs (data not shown).

To semi-quantify the amount of RNAi inhibition of endogenous TRIM mRNA knockdown, 5.0-fold serial dilutions were made of cDNA from the RT reaction of RNA extracted from siTRIM treated HeLa/CD4 cells (this work was done by Keith Aubin). Fig 6.2b shows that RNAi for TRIMs 1, 18 and 34 could knockdown their respective targets, however TRIM RNAi didn't knockdown the mRNA of a heterologous TRIM. TRIM knockdown is indicated by 5.0-fold less cDNA/RNA expression than a heterologous TRIM or the β -actin control (fig 6.2b).

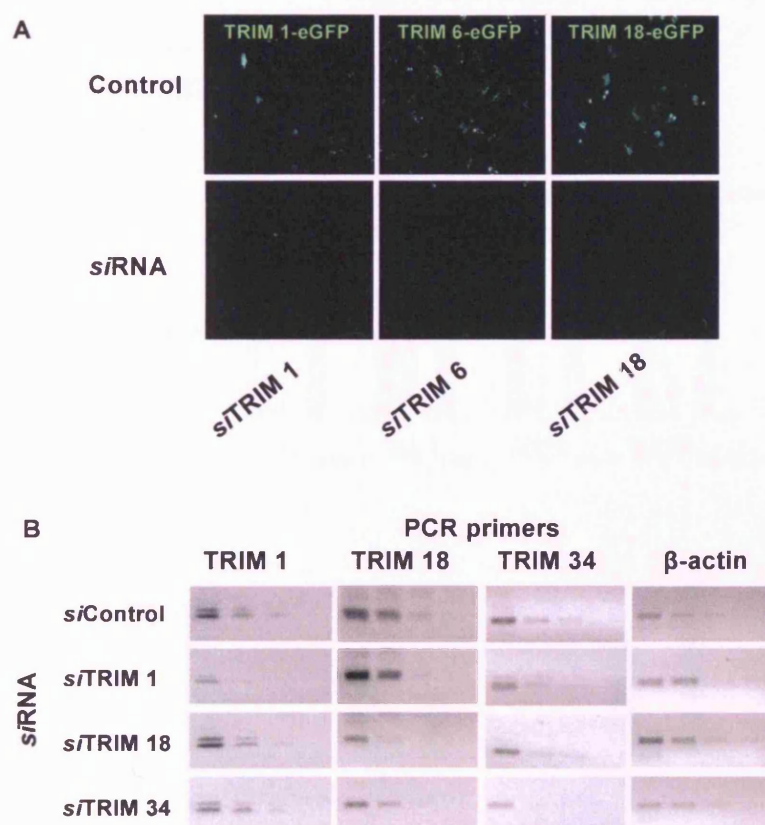


Figure 6.2. RNA interference knocks down the expression of tripartite motif proteins 1, 18 and 34 specifically and exclusively.

(A) The expression of eGFP-tagged TRIMs 1, 6, and 18 transfected into HeLa/CD4 cells is knocked down specifically by siRNA as visualised by epifluorescence. (B) TRIM 1, 18 and 34 endogenous mRNA transcript levels are down regulated by specific siRNAs. Five μ g total RNA from siRNA treated HeLa/CD4 cells was reverse transcribed and PCR was performed on 5.0-fold serial dilutions of cDNA. TRIM specific primers did not down modulate expression of other TRIMs or β -actin loading control. For

primer sequences see materials and methods. The results shown are representative of 3 independent experiments for (A) and (B).

6.2.3 Knockdown of tripartite motif proteins 1, 18 and 34 by RNA interference rescues restricted HIV-1 and HIV-2 infection in Lv2 restrictive HeLa/CD4 cells.

Fig 6.3b shows that there is little effect of any of the TRIM RNAi on the unrestricted viruses, HIV-1 NL4.3 and HIV-2 MCN, however TRIM 1 RNAi alone recovered the 10.0-fold restriction (10.0-fold in fig 6.1c) of HIV-1 89.6 in HeLa/CD4 cells. TRIM 1 RNAi rescued 15.0-fold of HIV-2 MCR restriction in HeLa/CD4 cells and when all 3 TRIMs were knocked down together the entire 25.0-fold restriction of MCR (compared to 20-fold of restriction in fig 6.1c) was recovered. Fitting with the Lv2 model, fig 6.3c shows that RNAi knockdown had little effect on restriction of MCR or 89.6 virus titrations in permissive U87/CD4/CXCR4 cells.

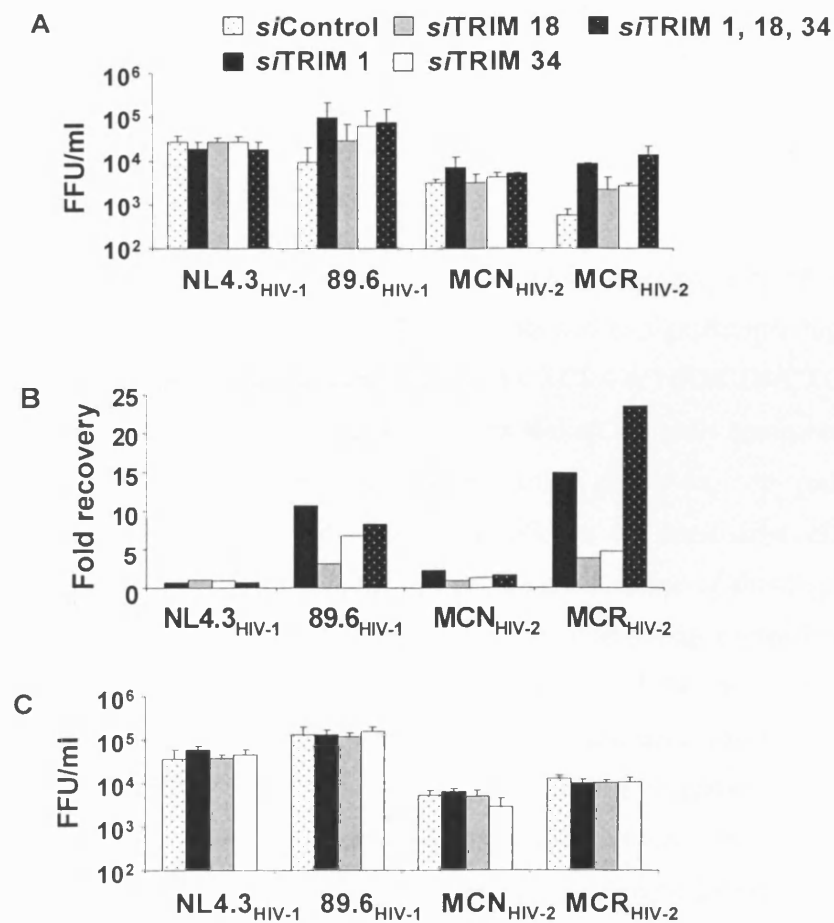


Figure 6.3. Knockdown of tripartite motif proteins 1, 18 and 34 with RNA interference rescues Lv2 restricted MCR and 89.6 infection in HeLa/CD4 cells.

(A) HeLa/CD4 and (C) U87/CD4/CXCR4 cells were transfected with RNAi oligomers directed against TRIM or irrelevant cellular protein and infected with HIV 2 days later, and fixed and stained for HIV infection 3 days later. (B) Fold recovery of HIV infection on HeLa/CD4 cells. The virus titre from infection on TRIM RNAi treated HeLa/CD4s was divided by the titre from the control RNAi HeLa/CD4 cells to obtain fold recovery. The results shown in (A) represent the mean of the results pooled from 7 independent experiments. The error bars in (A) represent the SDM of the pooled results from 7 independent experiments. The results shown in (B) are the product of 1 experiment but represent the results of 2 independent experiments and error bars represent the SDM.

6.2.4 Tripartite motif proteins 1, 18 and 34 are expressed to higher endogenous levels in restrictive HeLa/CD4 than in permissive U87/CD4/CXCR4 or NP2/CD4/CXCR4 cells.

To determine whether TRIMs 1, 18 and 34 were expressed endogenously in the permissive U87/CD4/CXCR4 and NP2/CD4/CXCR4 cell types I used specific polyclonal Abs to detect these TRIMs in HeLa/CD4, U87/CD4/CXCR4 and NP2/CD4/CXCR4 cells.

Figs 6.4a and b show the detection of TRIMs 1, 18 and 34 by western blot. Despite almost identical actin input, TRIMs 1, 18 and 34 are expressed to significantly higher levels in restrictive HeLa/CD4 than permissive U87/CD4/CXCR4 or NP2/CD4/CXCR4 cells. There are higher levels of TRIM 1 α expression in HeLa/CD4 cells compared to permissive NP2/CD4/CXCR4 and U87/CD4/CXCR4 cells (fig 6.4a, top panel). Difference in expression of TRIM 18 α and β between restrictive and permissive cells is the most apparent in that there is no detectable expression of either of these splice variants in NP2/CD4/CXCR4 or U87/CD4/CXCR4 cells despite strong expression in HeLa/CD4 cells (fig 6.4a, middle panel). Detection of the TRIM 34 splice variants yielded the α and 'short' isoforms in addition to an as yet uncharacterised 80 kDa band (fig 6.4a, bottom panel). The difference in expression of the α variant between HeLa/CD4 and permissive NP2/CD4/CXCR4 and U87/CD4/CXCR4 cells was most apparent. There is less expression of the short variant in permissive NP2/CD4/CXCR4

compared to HeLa/CD4 cells, however, like HeLa/CD4, U87/CD4/CXCR4 cells express high amounts of this variant (fig 6.4a).

The effect on TRIM 1 protein expression from TRIM 1 RNAi treated restrictive and permissive cells was determined. A 5.0-fold reduction in TRIM 1 mRNA production was seen in HeLa/CD4 cells upon TRIM 1 RNAi treatment and considerable knockdown was also seen with the TRIM 1-eGFP transfected construct in these cells, however the western blot protein band is only marginally smaller than the control band in fig 6.4b.

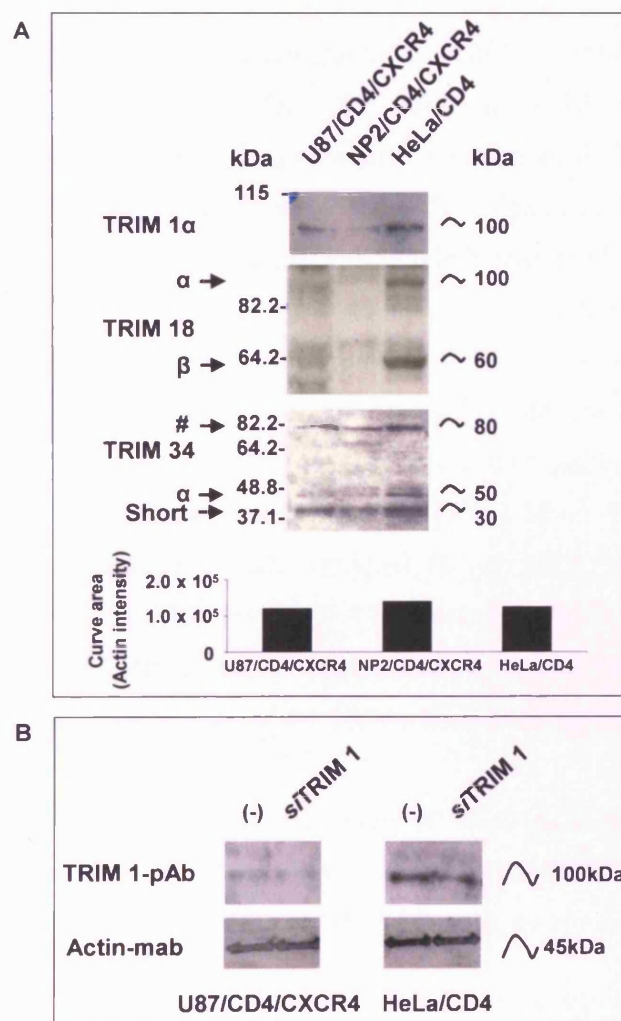


Figure 6.4. Tripartite motif proteins 1, 18 and 34, are expressed to higher levels in restrictive HeLa/CD4 than in permissive U87/CD4/CXCR4 and NP2/CD4/CXCR4 cells.

(A) Cell lysates were western blotted and probed with anti-TRIM 1, 18 and 34 polyclonal antibodies. Detection of actin with an actin mAb was used as an input control and band intensity was measured by densitometry, below (see materials and methods). Arrows denote position of detectable splice variants on western blots. The α , β and 'Short' isoforms are indicated and the long uncharacterised 80kDa TRIM 34 isoform is denoted as #. (B) Cell lysates from control and TRIM 1 RNAi treated cells were western blotted and probed with anti-TRIM 1 polyclonal antibody and anti-actin mAb as an input control. The figs shown are the product of one experiment but represent the results from at least 7 different western blots of each of the TRIM proteins.

6.2.5 Permissive cells expressing human tripartite motif proteins 1, 18 or 34 become restrictive.

The RNAi knockdown of TRIM expression (controls in fig 6.3) resulted in recovery of restricted HIV infection in restrictive HeLa/CD4 cells. If TRIMs 1, 18 and 34 are indeed responsible for Lv2 then it is important to recapitulate the restriction by expressing these candidate TRIMs in permissive cells and demonstrating restriction of Lv2 susceptible HIV. Fig 6.5c shows that HIV-1 89.6 infection in TRIM 1 β expressing NP2/CD4/CXCR4 cells is restricted 6.0-fold compared to infection on the parental NP2/CD4/CXCR4 cells. TRIM 18 β also restricted 89.6 by about 6.0-fold as well, and TRIM 34 α displayed weaker restriction activity at only 2.0-fold less infection than the NP2/CD4/CXCR4 parental cells. So it would appear that individually expressed TRIMs 1 and 18 effect the majority of restriction on HIV-1 89.6. Trim 18 β and 34 α expressing NP2/CD4/CXCR4 cells each restricted HIV-2 MCR by about 6.0-fold whereas TRIM 1 β expressing NP2/CD4/CXCR4 cells restricted MCR by 11.0-fold (fig 6.5c). HIV-1 NL4.3 and HIV-2 MCN were relatively unaffected by any of the NP2/CD4/CXCR4 cells expressing any of the TRIM constructs (fig 6.5c).

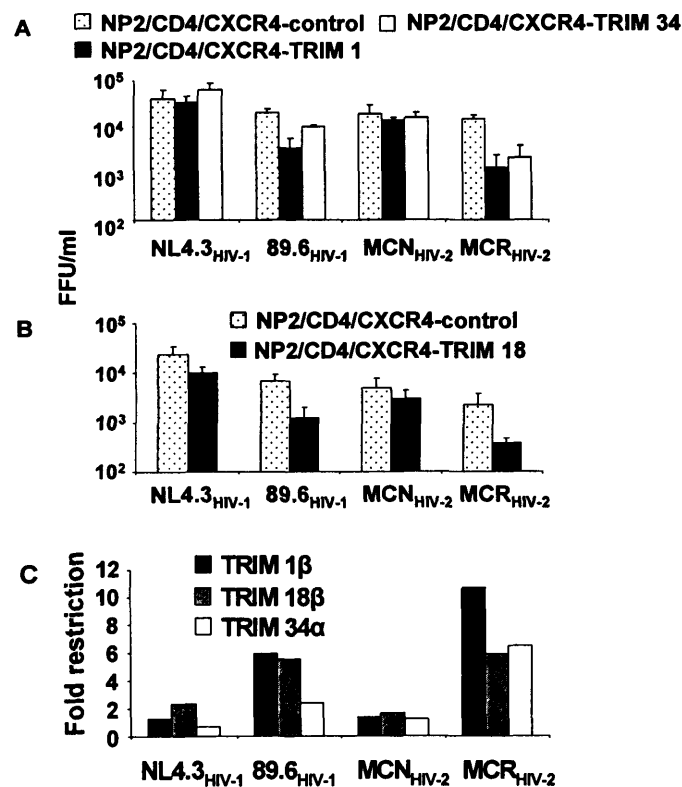
MCR(VSV) was titrated onto TRIM 1 β expressing NP2/CD4/CXCR4 cells alongside MCR. Fig 6.5g shows that TRIM 1 β restriction is bypassed in NP2/CD4/CXCR4 cells by a VSV Env, supporting the implication of TRIM 1 β in the Lv2 model of restriction.

6.2.6 The variable expression of tripartite motif proteins in permissive cells may explain the different restriction activities of the over-expressed tripartite motif proteins.

Cell lysates from TRIM 1 β , 18 β and 34 α expressing NP2/CD4/CXCR4 cells were western blotted and probed with TRIM 1, 18 and 34 specific antibodies (fig 6.5d).

The detection of TRIM 18 β and 34 α expression by western blot is less apparent. Figs 6.4a and 6.5e show the differential expression of TRIM 18 α and β in HeLa/CD4 cells compared to the parental NP2/CD4/CXCR4 cells. Additional expression of TRIM 18 β can be seen in NP2/CD4/CXCR4 cells, transduced and stably expressing this protein over the parental NP2/CD4/CXCR4 (fig 6.5e; the two arrows). Although the over-expressed band sizes are not the expected sizes of approximately 60 and 100 kDa for TRIM 18 β and α , respectively, as seen in restrictive HeLa/CD4 cells.

The over-expression of TRIM 34 reveals one band at approximately 115 kDa on the western blot (fig 6.5f). This size is reminiscent of the size of the over-expressed TRIM 18, however there is only one band at 115 kDa on the TRIM 34 α western blot (fig 6.5f).



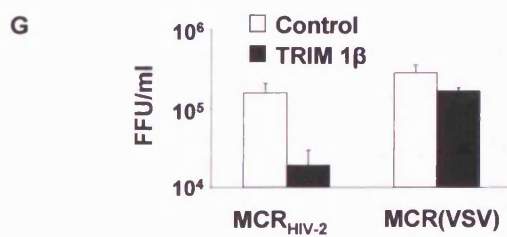
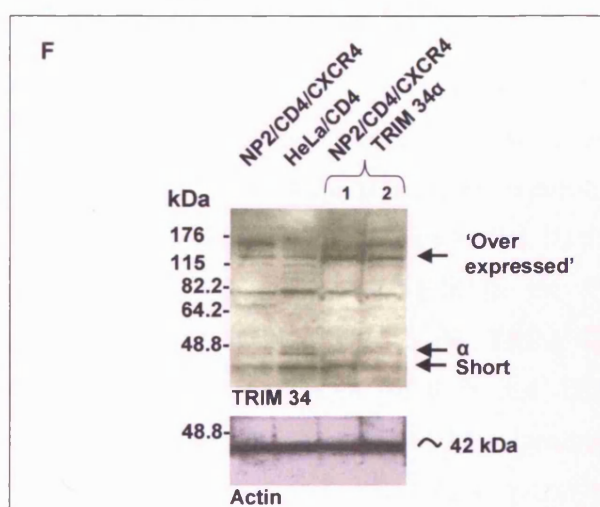
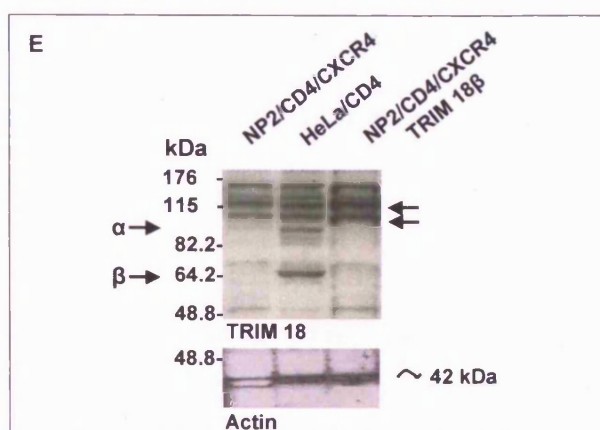
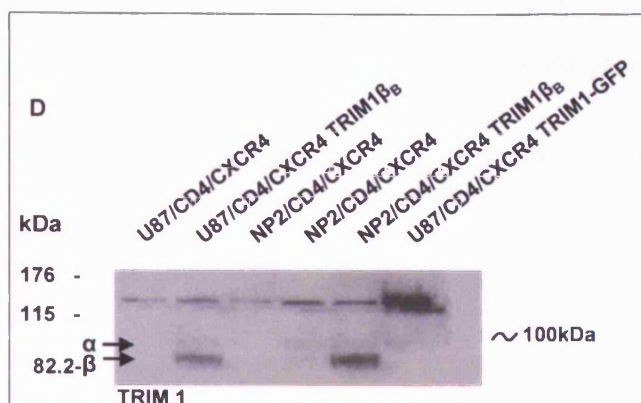


Figure 6.5. Permissive NP2/CD4/CXCR4 cells can be made restrictive to HIV-1 89.6 and HIV-2 MCR infection by expressing tripartite motif proteins 1 β , 18 β and 34 α .

(A) TRIMs 1 β , 34 α and (B) 18 β were cloned from HeLa/CD4 cells, sub cloned into an MLV retroviral vector, then transduced into permissive NP2/CD4/CXCR4 cells. Cells were infected with HIV or (G) HIV(VSV) pseudotype, fixed and stained for infection 3 days later. Error bars represent the SDM. (C) Fold restriction was calculated by dividing the infection on control NP2/CD4/CXCR4 cells by the TRIM transduced cells. All three TRIMs are shown together. (D-F) Lysates from U87/CD4/CXCR4 and NP2/CD4/CXCR4 cells stably expressing (D) TRIM 1 β , (E) TRIM 18 β or (F) TRIM 34 α were western blotted and probed with anti-TRIM 1, TRIM 18, or TRIM 34 pAbs, respectively. All of the cell lysates in (D), (E) and (F) were western blotted and probed with an actin mAb as an input control. The upper arrow in (D) indicates the position of TRIM 1 α and the lower arrow indicates the position of TRIM 1 β . The figs shown are the product of one experiment but results represent the results of at least 4 independent experiments.

6.2.7 The tripartite motif protein 1 β -SPRY (butyrophilin 30.2) domain possesses the majority of restriction activity of tripartite motif protein 1 β .

A research assistant in the lab, Keith Aubin cloned TRIM 1 β without a SPRY domain (TRIM 1 β Δ SPRY). The antiviral activity of TRIM 5 α has been reported to reside, primarily, in the SPRY (B30.2) domain at the C-terminal end of the protein (Stremlau et al., 2004; Stremlau et al., 2005). In TRIM 1 β this corresponds to aa position 397 to 685, the last 288 amino acids. NP2/CD4/CXCR4 cells stably expressing TRIM 1 β - Δ SPRY lose the majority of their restriction activity towards HIV-1 89.6 and MCR. Fig 6.6b shows that restriction of 89.6 goes from 6.0-fold in the TRIM 1 β expressing NP2/CD4/CXCR4 cells to 2.0-fold restriction in the TRIM 1 β - Δ SPRY expressing NP2/CD4/CXCR4 cells. The restriction of MCR in the TRIM 1 β - Δ SPRY cells decreases to 4.0-fold from 11.0-fold in the TRIM 1 β expressing NP2/CD4/CXCR4 cells. As was seen with TRIM 1 β in fig 6.5c, the TRIM 1 β - Δ SPRY had little effect on the infection of HIV-1 NL4.3 or HIV-2 MCN in NP2/CD4/CXCR4 cells (fig 6.6b)

Fig 6.6c shows the level of expression of TRIM 1 β - Δ SPRY in NP2/CD4/CXCR4 cells with an anti-HA mAb. TRIM 1 β - Δ SPRY was cloned with an HA tag at the C-terminus because the TRIM 1 β antibody used to detect TRIM 1 β over-expression in fig 6.5d recognises a motif in the SPRY region of TRIM 1. Therefore, in this system, direct

comparisons between western blots of over-expressed TRIM 1 β and TRIM 1 β - Δ SPRY are difficult to make. The western blots in figs 6.5d and 6.6c show that TRIM 1 β and TRIM 1 β - Δ SPRY proteins, respectively, are over-expressed in the parental NP2/CD4/CXCR4 cells to roughly similar levels.

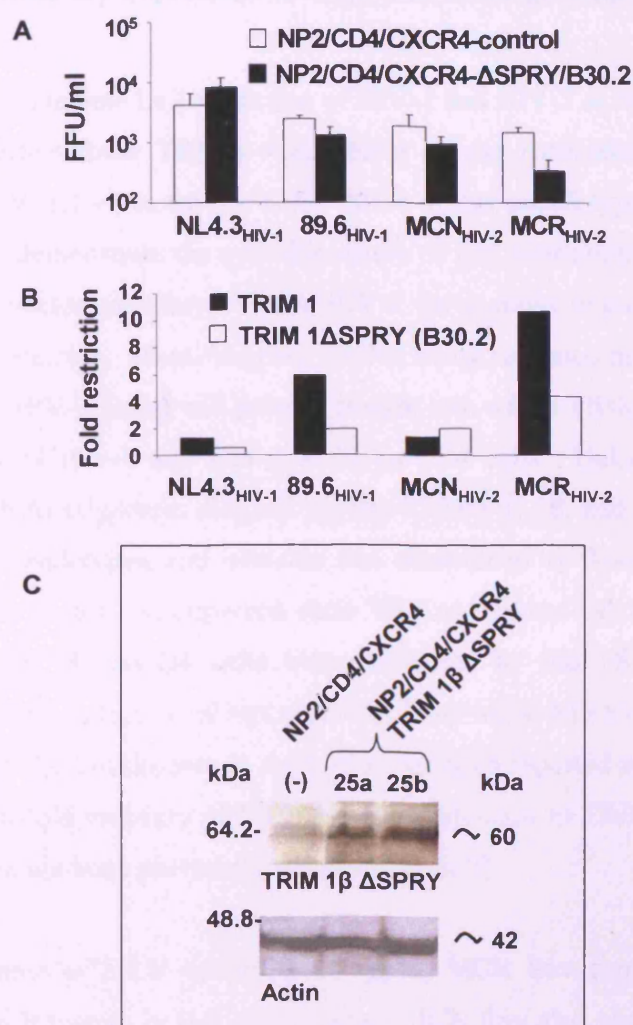


Figure 6.6. Tripartite motif protein 1 β without a SPRY (butyrophilin 30.2) domain (tripartite motif protein 1 β Δ SPRY) has limited restriction activity against HIV-1 and HIV-2 compared to full-length tripartite motif protein 1 β .

(A) TRIM 1 β Δ SPRY was stably expressed in NP2/CD4/CXCR4 cells. Cells were challenged with HIV and fixed and stained for infection 3 days later. (B) The fold restriction of HIV infection on TRIM 1 NP2/CD4/CXCR4 cells calculated from (A) compared to infection on TRIM 1 β Δ SPRY NP2/CD4/CXCR4 cells. Fold restriction was obtained by dividing the infectious titre on control NP2/CD4/CXCR4 cells by the titre on TRIM 1 β Δ SPRY NP2/CD4/CXCR4 cells in (A). (C) Cell lysates

from NP2/CD4/CXCR4 cells stably expressing TRIM 1 β Δ SPRY were western blotted and probed with an anti-HA-tag mAb or an anti-actin mAb as an input control.

6.2.8 Infection by HIV-2 envelope pseudotypes of MLV can be recovered in HeLa/CD4 cells when the expression of tripartite motif proteins 1, 18 and 34 is knocked down.

TRIMs 1, 18, and 34 mediate Lv2 restriction of HIV-1 and HIV-2 as described above. I determined what effect these TRIMs would have on the replication of HIV-2 Env pseudotypes of MLV in Lv2 restrictive cells. MLV vector pseudotypes were used as a tool in Chapter 5 to demonstrate the saturable nature of Lv2 restriction. Saturable doses of MLV-N and B vector pseudotypes with HIV-2 Envs could recover Lv2 restricted HIV-1 and HIV-2 infection. Identifying the TRIMs being saturated by incoming MLV-N and B (that have HIV-2 Envs) will provide insight into which TRIMs are responsible for Lv2 restriction of HIV-1 and HIV-2 in HeLa/CD4 cells. HeLa/CD4 cells were transfected with RNAi oligomers directed against TRIMs 1, 18, and 34, infected with HIV-2 Env-MLV pseudotypes, and infection was determined by flow cytometry of the MLV-eGFP reporter vector. As expected, since VSV overcomes Lv2 restriction, MLV-B(VSV) infection of HeLa/CD4 cells was unaffected by the TRIM 1, 18 or 34 knockdowns (fig 6.7f). Infection of HeLa/CD4 cells by MLV-N(VSV) was rescued by 4.0-fold by the TRIM 1 knockdown in fig 6.7f as has been reported previously (Yap et al., 2004). The 8.0-fold recovery of MLV-N(VSV) infection in TRIM 34 knockdown HeLa/CD4 cells has not been previously reported (fig 6.7f).

In chapter 5 (titration of MLV vector pseudotypes) MCR Env confers restriction to MLV cores (N and B-tropic) in HeLa/CD4 cells. MCN Env also confers restriction to MLV-B cores (but to a lesser extent) and no further restriction to MLV-N capsid cores. I postulated that the lack of MLV-N restriction was because it is already restricted by Ref1 in human cells. Figs 6.7a to d show that MLV-N(HIV-2) pseudotypes are more susceptible to recovery with RNAi TRIM knockdown in HeLa/CD4 cells than the MLV-B(HIV-2) pseudotypes.

The TRIM knockdown cells recovered MLV-N infection differently, depending on the Env provided. Infections by both MLV-N(MCR) and MLV-N(MCN) were rescued by the TRIM 1 knockdowns by 6.8 and 4.4-fold, respectively, in figs 6.7b and d. The TRIM 18 knockdown didn't affect the MLV-N(MCN) infection but recovered MLV-N(MCR) infection by 3.0-fold (fig 6.7b), which is consistent with the restriction of HIV-2 MCR and HIV-1 89.6 by TRIM 18 in fig 6.3b. The co-knockdowns of all three TRIMs is additive for MLV-N(MCN) but not MLV-N(MCR) (figs 6.7b and d).

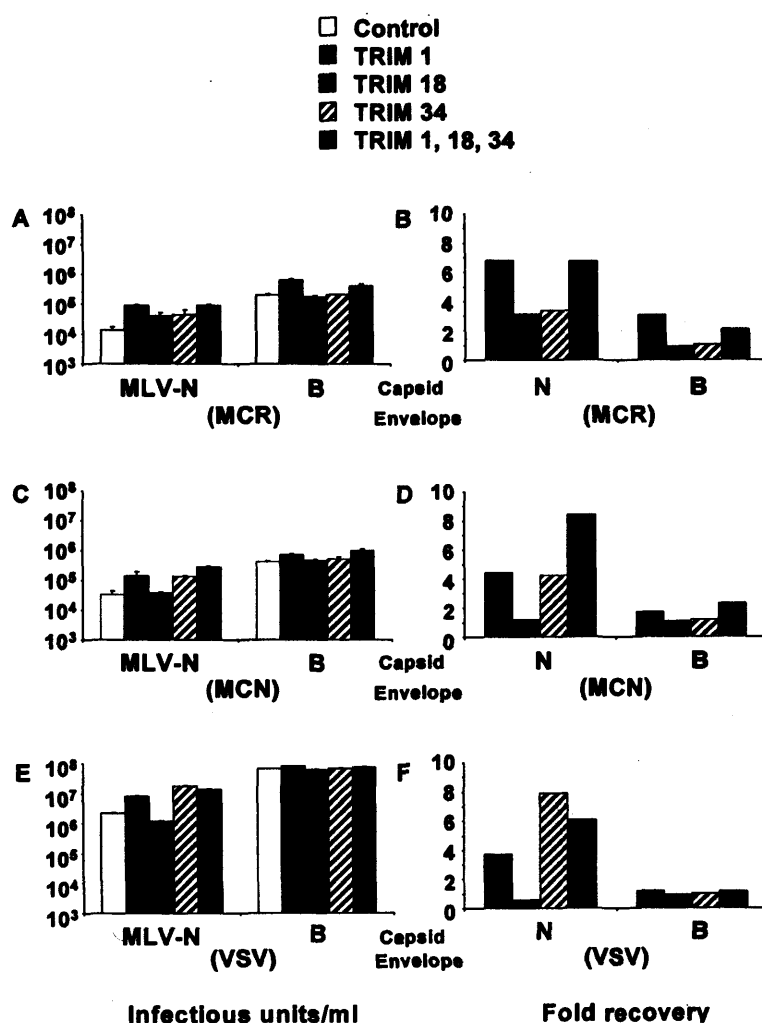


Figure 6.7. MCR and MCN envelope pseudotypes of N and B-tropic MLV are rescued on restrictive HeLa/CD4 cells knocked down for tripartite motif proteins 1, 18, and 34.

(A-F) HeLa/CD4 cells treated with control, TRIM 1, 18 or 34 RNAi and all three pooled together were infected with MLV-N and B pseudotypes with MCR, MCN, and VSV Envs. (A, C, E) EGFP positive cells were determined by flow cytometry, and infectious units per ml were calculated. (B, D, F) Fold recovery of pseudotype infection with TRIM RNAi knockdown. The TRIM RNAi infection was divided by the control RNAi infection to obtain fold recovery. Results are representative of 6 independent experiments. Error bars represent SDM.

6.3 Discussion:

This chapter demonstrates that TRIMs 1, 18 and 34, mediate Lv2. By gene silencing in restrictive HeLa/CD4 and by rendering permissive NP2/CD4/CXCR4 cells restrictive I show that most Lv2 restriction activity lies in the activity of TRIM 1. Furthermore, the SPRY domain of TRIM 1 is implicated as an important determinant of antiviral activity of TRIM 1 β . This chapter also shows that the difference in restriction activity between Lv2 permissive and restrictive cells lies in the expression of monomeric TRIM proteins in restrictive cell types compared to permissive ones. Infection of TRIM knockdown cells with HIV-2 Env pseudotypes of MLV-N and B cores suggests that the MLV capsid cores are 'hit' by different TRIMs when delivered into one compartment by HIV-2 Envs than when delivered to another compartment by a VSV Env. Thus the compartmentalisation models of TRIM proteins (Reymond et al., 2001) and Lv2 retroviral restriction (Schmitz et al., 2004) are mutually supportive.

Some endogenous expression of TRIM 1 in permissive cells is detectable. The western blots show trace amounts of TRIM 1 protein expression, however significant levels of recovery of MLV-N(VSV) with TRIM 1 RNAi, in permissive cells, suggests that TRIM 1 is normally expressed in these cells as well as restrictive ones and that only small amounts are required to inhibit MLV-N.

PH-dependent VSV-G delivers its contents from an acidic compartment whereas pH-independent HIV can fuse outside acidic compartments. Acid-dependent VSV-G can only fuse in a low pH compartment (Mannen et al., 1982). TRIM 5 α activity is associated with this pathway of entry because MLV-N(VSV) is restricted in a Ref1-dependent manner. However, in chapter 5 the knockdown of TRIM 5 α recovered the potency of MLV-N(HIV-2) vectors to saturate-out Lv2 restriction suggesting a more ubiquitous localisation of TRIM 5 α in the cell than Lv2 activity.

I showed that individual TRIMs are responsible for restriction of HIV-1 89.6 and HIV-2 MCR. However, I have not shown what happens when all of these proteins are over-expressed simultaneously. The over-expression of these three proteins together may very well result in recapitulation of the full Lv2 restriction.

The ability to target different viruses (via the different viral entry pathways) may, in part, lie in the differing nuclear and cytoplasmic localisations of the 69 TRIM gene products (Nisole et al., 2005), that can form homo and hetero-oligomers in different cellular compartments (Reymond et al., 2001). This particular chapter is especially pertinent to the Lv2 model of restriction, because Lv2 restriction (Schmitz et al., 2004), like TRIM localisation (Reymond et al., 2001), is a model based on compartmentalisation. Depending on the virus and target cell type, virus Envs target different cellular entry pathways (Marchant et al., 2005).

Chapter 7

Summary and future directions

7.1 Summary

In chapter 3 I observed that although HIV-1 and HIV-2 both infect PBMC and MDM the replication kinetics of HIV-2 in MDM are different. HIV-2 primary isolates could enter MDM and reverse transcribe efficiently but entered a latent state after an initial burst of replication.

The V3 loop of Env is a MDM cell tropism determinant in HIV-1 and I show that this is also true for HIV-2, although weaker. The correlation suggests that there are other factors associated with HIV-2 infection of MDM. One HIV-2 isolate in particular, prCBL 23, demonstrated efficient infection of PBMC but lacked the ability to infect MDM. In chapter 4 I show that the role of Env in Lv2 restriction may be explained by its delivering susceptible cores into restrictive compartments via an endocytic pathway.

I demonstrate the saturable nature of the Lv2 restriction for HIV-1 and HIV-2 in chapter 5 and in chapter 6 I demonstrate that TRIMs 1, 18, and 34 mediate Lv2.

7.2 Future directions

To better understand the role that restriction plays in viral infection it would be interesting to investigate the tissue specific expression of viral restrictions and which types of viruses are targeted by these factors. Work resulting from this thesis might also involve investigating the possible localisation of TRIMs in endocytic compartments. Arf6 was implicated as a restrictive compartment thus this may be a region of high TRIM density. Site directed mutagenesis would also be a useful tool to relocalise TRIMs to other cellular compartments and observe the effect on restriction to further investigate the compartmentalisation model of restriction.

Publications resulting from this thesis

Marchant D, Neil SJ, Aubin K, Carter G, McKnight A. Multiple cytoplasmic human tripartite motif proteins terminate human immunodeficiency virus replication. **Nature.** *Submitted, in review.*

Marchant D, Neil SJ, McKnight Á. Latency of HIV-2 in Macrophages. **Journal of General Virology.** *Accepted, October 2005.*

Marchant D, Neil SJ, Aubin K, Schmitz C, McKnight Á. The route of viral entry determines the susceptibility of HIV-1 and HIV-2 to Lv2 restriction. **J Virol.** 2005 Aug;79(15):9410-8.

Schmitz C, **Marchant D, Neil SJ, Aubin K, Reuter S, Dittmar MT, McKnight Á.** Lv2, a novel post-entry restriction, is mediated by both capsid and envelope. **J Virol.** 2004; 78(4):2006-16.

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